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14. ABSTRACT

The Crk adaptor proteins (CrkI, CrkII and CrkL) play an important role during cellular signalling by mediating the formation of protein-protein complexes and are involved in cellular migration, invasion, and adhesion. Targeting CrkI and CrkII in breastcancer cell lines by RNA interference demonstrated that loss of Crk expression corresponded with a significant decrease in cellmigration and invasion. This implies that Crk adaptor proteins play an important role in integrating signals for migration and invasion of highly malignant cancer cell lines. As migration and invasion are important components of the metastatic cascade, future work includes stable knockdown of Crk in breast cancer cell lines and performing in vivo metastasis assays. Furthermore, mouse models over-expressing CrkI/II result in delayed ductal outgrowth. MMTV-CrkII mice display enhanced branching and leads to tumour development. This has important implications as we have shown elevated levels of Crk are observed in human breast cancer. This project may provide information, which could be used to develop effective treatments for breast cancer, as well as other cancer types.

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Role of Crk adaptor proteins in cellular migration & invasion in human breast cancer

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INTRODUCTION

The Crk protein was originally identified as the oncogene fusion product of the CT10 chicken retrovirus (v-Crk) (1). Cellular homologues of v-Crk, include c-Crk, which encodes two alternatively spliced proteins (c-CrkI and c-CrkII), and c-CrkL. Crk proteins are composed of one Src homology 2 (SH2), and one or two Src homology 3 (SH3) domains (2). The Crk adaptor proteins (CrkI, CrkII and CrkL) play an important role during cellular signalling (of processes such as migration, invasion, & adhesion) by mediating the formation of protein complexes. Previous work in our lab has shown that the over-expression of Crk proteins promotes an invasive phenotype, regardless of upstream signalling implicating a role for Crk proteins in cancer (3, 4). Moreover, Crk proteins are over-expressed in multiple types of cancer, including lung carcinoma, glioblastoma, and breast cancer (5, 6, 7). Although many studies have focused on the over-expression of Crk being important for many cellular processes, their significance in inherently motile cancer cells still remains elusive. Furthermore, much of what has been proposed about Crk proteins in epithelial cells is based upon in vitro over-expression studies or the use of dominant interfering mutants of CrkI/II or CrkL. These approaches, while informative, are limited by the challenges intrinsic to over-expression systems and these in vitro assays do not truly reflect the complexity of an in vivo response. Thus the goal of our study was to examine if Crk adaptor proteins are truly responsible for mediating signals required for migration and invasion by validating how loss of Crk would affect these processes. Through the use of short interfering RNA (siRNA) silencing techniques and in vivo model systems, I will establish experimental models to formally test the hypothesis that *Crk proteins play an important role in cellular migration, invasion and metastasis, through their ability to recruit specific protein-protein complexes.* Our study on Crk adaptor proteins has the potential to identify some of the molecular events, which can occur during metastasis. Furthermore, since Crk proteins are over-expressed in human breast cancer, I will establish mouse models to test the hypothesis that *over-expression of Crk can promote breast tumorigenesis.* This may provide information, which could be used to develop effective treatments for breast cancer.

BODY:

(1) Role of Crk in cellular migration and invasion in various types of cancer

Several studies have suggested that Crk mediates cellular migration and invasion downstream of various stimuli. *I tested the hypothesis that Crk functions as a central adaptor protein that integrates upstream signals for cell migration and invasion by examining how the loss of Crk expression affects these processes in highly invasive cancer cell lines.* Using RNAi, I targeted CrkI, CrkII and CrkL in highly malignant breast cancer cell lines (MDA-435s and MDA-231) and found that the loss of Crk was associated with a decrease in cellular invasion and migration (7). In addition, migration was significantly decreased even after stimulation with HGF, a known stimulant of cellular migration (7). These results demonstrate that Crk adaptor proteins play an important role in the promotion of signals for cell migration and invasion in human breast cancer cell lines irrespective of the upstream regulatory events that promote the invasive response. These results are now published in Molecular Cancer Research (See Appendix 1) (7).

I extended these studies by analyzing Crk dependent migration and invasion (using a siRNA-mediated approach) on a panel of breast cancer cell lines available in the lab (ie. BT-474, SKBR3, BT-20, BT-549) in order to gain a better understanding of the precise role of Crk adaptor proteins in breast cancer. Certain breast cancer cell lines (BT-474, SKBR3) were unable to either migrate or invade, suggesting that they are not highly aggressive. Although Crk proteins are present in these cell lines, the ability of Crk to inhibit these processes was unable to be tested by this method. Indeed, it has been recently shown by other groups that these cell lines are not highly invasive or motile (8). More interestingly, gene expression profiling on a wide panel of human breast cancer cell lines have distinguished breast cancer cell lines into two distinct classes – luminal and basal, results similar to those obtained from human breast cancer patients (8, 9). The cell lines which were poorly migratory and invasive (BT-474, SKBR3) fell into the luminal category, which is associated with good prognosis (8, 9).

Other cell lines tested (BT-20, BT-549) were considered to be of a basal phenotype, which is associated with poor prognosis and decreased survival in human patients (8, 9, 10). When Crk siRNA was transfected into basal cell lines, inhibition of cellular migration and invasion was quite significant. For instance, invasion was inhibited anywhere from 30-90% depending on the cell line examined (See Appendix 2). These data demonstrate that Crk adaptor proteins play an important role in integrating signals for

migration and invasion of highly malignant cancer cell lines. Furthermore, this information, coupled with the known biological properties of Crk, identifies a potential role for Crk in metastasis. This has important implications as we have shown elevated levels of Crk are observed in human breast cancer (7). Studies by Tanaka and colleagues have examined the role of Crk in ovarian cancer and have found that Crk is important for cellular migration and invasion through RNAi-based studies as well (11). They have found that loss of Crk inhibits tumor formation and metastatic growth. However, their metastatic assays fail to recapitulate all aspects of the metastatic cascade, and thus, the precise role of Crk during metastasis has yet to be revealed. Furthermore, the metastatic ability of ovarian cancer is different from breast cancer, thus the precise role of Crk in breast cancer metastasis still needs to be addressed.

(2) Role of Crk adaptor proteins in metastasis

Since Crk proteins are over-expressed in various types of cancer, including breast cancer, and Crk appears to play a key role in processes required for metastasis, *I hypothesize that Crk proteins play a critical role in the metastatic spread of human breast tumors.* To test this hypothesis, I will utilize both cell-based studies and animal models to better understand the role of Crk in the metastatic cascade. To examine the role of Crk in metastasis, I will inject MDA-231 breast cancer variants expressing stable Crk or CrkL shRNA into nude mice and determine the number of metastases formed from "knockdown" cells versus control cells. The variant cell line, MDA-231 1833-TR has a higher propensity to form bone metastases compared to the parental cell line when injected into the left cardiac ventricle (12). The parental cell line, MDA-231 TR has the ability to spontaneously form lung metastases when injected into the tail vein or the mammary fat pad (12, 13). If I am successful in preventing or lessening the formation of metastases in orthotopic transplant models, I can conclude that Crk plays an important role in the metastatic cascade of breast cancer.

At the present time, I have designed stable Crk shRNA for these experiments and have successfully ligated the shRNA oligos into the pSUPER and pSUPERIOR vectors, but decided to focus on stable knockdown using the pSuper vector. The shRNA sequences are derived from the sequences used in the transient siRNA assays from the previous chapter. Although these vectors have previously been shown to give efficient knockdown of their target genes, the exact mechanism by which these vectors enter the RNAi pathway and thus, elicit such a response is not well known (14). Thus, I have also obtained shRNA targeting CrkI/II or CrkL in pSM2 vectors, which contains a pol III promoter and is modeled after microRNA, in that the constructs are recognized by Drosha, Dicer and RISC (15). These shRNA vectors are supposed to give more efficient knockdown compared to previous shRNA vectors (16). Finally, I cloned the shRNA sequences from pSM2 into the LMP (pol II promoter) vector, which was recently found to give more robust knockdown than the previous pSM2 vectors (17). Discussion with members from Scott Lowe's lab at Cold Spring Harbour have described the LMP vectors as being much more reliable than the pSM2 constructs. While I cloned the shRNA constructs into the LMP vectors, I infected MDA-231 cells with pSM2 and pSuper constructs.

It took several months to achieve stable knockdown. The MDA-231 cell lines are difficult to transfect and several transfection protocols were initiated before switching to a viral mediated approach. Furthermore, previous publications have suggested that transfection of pSM2 vectors is possible, however, upon discussion with other scientists who work with these constructs, it was suggested that viral infection was a much better option. Finally, the Phoenix packaging cell line originally used for the viral infections appeared to have lost its packaging components, which resulted in very low viral titres. The 293 VSV cell line was used instead and gives a much higher titre than the previous line used. Thus, after months of optimizing the stable knockdown system, a protocol has finally been established and preliminary results are positive.

I obtained stable clones expressing these shRNA and a few clones were successful at achieving stable and specific knockdown of Crk or CrkL (Appendix 3). Whole cell populations of pSuper-Crk shRNA results in stable knockdown of CrkI and CrkII. No knockdown was achieved with stable populations of CrkL, however individual clones may have yielded better results. The pSM2 vectors were not as promising as the pSuper constructs. Ten clones from each of the three shRNA constructs were tested of which only one clone gave slight knockdown. No knockdown was achieved using a pSM2 construct targeting CrkI/II. Overall, these shRNAs do not inhibit their respective targets (CrkI/II, CrkL) as robustly as transient siRNAs. This may be due to the fact that transient transfection of siRNA duplexes results in a higher percentage of duplex

intake, which results in increased knockdown. Stable knockdown was achieved through viral infections, in which one copy of each vector is inserted into each cell. However, it is possible that screening of additional individual clones may result in a more pronounced phenotype.

Currently, these shRNA constructs (pSuper, pSM2, LMP) are being used to infect the MDA-231 cell line variants (MDA-231 1833 TR, MDA-231 parental TR) obtained from Dr. Peter Siegel (12, 13). Preliminary results looking at whole cell populations of LMP constructs in MDA-231 1833 TR cells suggests that the LMP shRNA constructs targeting CrkL can induce knockdown, albeit with a slight knockdown of CrkI/II as well (Appendix 3). I hope that the clones can be screened for knockdown by the end of March and that shRNAs targeting CrkI/II and CrkL will be stably expressed and induce robust, specific knockdown in these cells. Before injecting these cell lines into mice, the ability of these Crk shRNA constructs to decrease migration and invasion will be tested in vitro using the boyden chambers described in chapter one. This will ensure that stable clones which have specific knockdown of Crk also inhibits migration and invasion of these cell lines.

I anticipate that Crk ablation will act to prevent or lessen the formation of bone and lung metastases in these models. If this is true, these experiments will formally establish that Crk plays an important role in the metastatic cascade of breast cancer. No prior data exists suggesting that Crk regulates these processes for breast cancer metastasis. To confirm that Crk proteins are indeed modulating the metastatic phenotype, rescue experiments will be performed in which cDNA expressing CrkI, CrkII or CrkL will be stably expressed into these MDA-231 cell variants to determine if the phenotype is recapitulated. This has important implications, as most studies that examine the Crk protein (which includes the isoforms CrkI and CrkII) never specify which of these two proteins are required for the migratory or invasive phenotype.

(3) To examine Crk dependent signaling pathways involved in cancer cell migration and invasion

Although I have demonstrated that Crk adaptor proteins act as key integrators for cellular migration and invasion, the exact mechanism by which Crk mediates such pathways has not been shown. Previous studies by Tanaka and colleagues have shown that loss of Crk leads to decreased Rac activation, which is required for cellular migration, however no further studies have been published detailing how the loss of Crk affects migration and invasion of inherently motile cancer cells (11). Originally, we had proposed to look at the role of Crk downstream from integrins, CD44 and various growth factors. However, the scope of that chapter was quite wide, thus we decided to adopt an unbiased approach to study how Crk regulates tumor progression. In this study, I will utilize microarray analysis to gain insight into the Crk dependent molecular mechanisms underlying migration and invasion of MDA-231 breast cancer cells. Over-expression of Crk can lead to the activation of various transcriptional pathways (AP-1, SRE-1) and Crk is a known activator of JNK and Rac, which can induce transcription (18, 19). Microarray analysis will allow me to identify the extent to which Crk is required for the regulation of genes involved in cellular migration and invasion and hence, determine the specific signals mediated by Crk. Breast cancer cell lines whose cellular migration and invasion are Crk dependent (i.e. MDA-231) will be transiently transfected with Crk siRNA, CrkL siRNA, and non-targeting siRNA and subjected to array analysis. A major advantage of this system, which distinguishes it from previous knockout approaches, is that the cells do not have to grow for long periods without Crk, and will thus, not have the opportunity to accumulate mutations or adjust expression levels of genes that might compensate for the loss of Crk. Multiple siRNAs will be used in this experiment to control for potential off-targeting effects caused by siRNA duplexes (20). Finally, the activation of downstream signals of Crk will also be examined (ie. Rac, JNK) to determine if loss of Crk impairs their activation. By carrying out these experiments, it may help us link the novel target genes Crk affects to a specific pathway Crk is known to activate (ie. Rac, JNK, Rap). The goal of the research is to determine a specific Crk signature, which is a group of genes regulated by Crk proteins.

Furthermore, microarray analysis of cells over-expressing Crk in MDA-231 cell lines will be performed as well. It can be assumed that genes down-regulated when Crk is lost will be up-regulated when Crk is over-expressed. The Crk “signatures” derived from this study could then be compared to human breast cancer array data. For instance, a division of Dr. Morag Park’s lab, the Breast Cancer Functional Genomics

Group (BCFGG), have generated gene expression profiles from laser capture microdissected tumor and matched-normal breast epithelium. For each patient whose tumor has been analyzed by microarray analysis, a 10-year follow-up history has been documented in a database developed by our lab. By comparing Crk–mediated profile to the BCFGG data on the same microarray platform, as well as to publicly available human data sets (ie. Van't Veer, Sorlie/Perou), we may be able to correlate Crk dependent signatures to specific breast cancer subtypes and to prognosis.

Since microarray analysis provides a large amount of experimental data, data sets will be filtered to the top over-expressed and under-expressed genes (with a fold change above 2) and the expression level of a subset of genes will be confirmed by quantitative real-time PCR and Western blot analysis. The role of these genes in tumor progression will be further characterized using traditional biochemical assays, such as RNAi, immunoprecipitation, Western blot analysis, dominant negative mutants, and immunofluorescence. Thus, this study has the potential to elucidate novel mechanisms by which Crk mediates essential components of tumor progression.

Several different siRNA duplexes targeting CrkI/II or CrkL has been ordered and future work will be to isolate RNA from MDA-231 cell lines expressing these different duplexes. Since optimization of the siRNA protocol has been established, it is believed that this protocol should run smoothly. Furthermore, the Park lab has previously established microarray protocols, so expertise in this area is available. I hope to start this aspect of my project in the upcoming months. At the present time, I have been trying to optimize Rac assays in order to test if loss of Crk inhibits Rac activation because confirming the downstream effectors affected by loss of Crk may determine the exact mechanism by which Crk affects transcription of certain target genes.

(4) Role of Crk adaptor proteins in the development of breast cancer using transgenic mice

To examine *the importance of Crk adaptor proteins in tumor progression*, I will use a transgenic mouse model, in which Crk proteins are over-expressed in the mammary epithelium. I have created constructs for CrkI, CrkII and CrkL, which allows the over-expression of these proteins through the hormonally responsive MMTV promoter. At the present time, MMTV-CrkII transgenic mice have been generated and have been genotyped and show strong transgene expression. The Crk protein can be detected through immunohistochemistry, RT-PCR (using primers specific for the transgene) and Western blot analysis (Appendix 4). Of the 7 founder lines, 2 lines had problems breeding. Of the five remaining lines, 2 were kept for further analysis. Tumor development is being monitored in both virgin and multiparous females, with a combined tumor incidence of 20% thus far and a tumor latency of approximately one year (Appendix 5). These initial results are promising, as many other adaptor and scaffold proteins over-expressed in the mammary gland were unable to induce tumorigenesis (ie. MMTV-Grb2, MMTV-p130Cas, MMTV-Gab2) (21, 22, 23). The CrkII tumors have been transplanted into FVB mice and cell lines have been established. Cell lines have been re-injected into mice to examine metastatic potential (via tail vein injection) as well as primary tumor growth and metastasis via mammary fat pad injection. One CrkII tumor has been identified as a spindle cell carcinoma, which is thought to be induced by an epithelial-to-mesenchymal transition (EMT). As previously shown in our lab, Crk proteins have the ability to induce EMT (4). The tumor is positive for CrkII via immunohistochemistry and staining for smooth muscle actin is almost identical to CrkII staining (Appendix 6). Staining for other EMT markers such as vimentin is ongoing. Another CrkII tumor has been identified as squamous adenocarcinoma, with a high mitotic index and the presence of cytokeratin pearls (Appendix 6). The pathology of the CrkII tumor is similar to MMTV models in which the Wnt pathway is over-expressed, suggesting that it may be a basal phenotype, which is often associated with poor prognosis in human cases (24). Staining was positive for CK14+ and smooth muscle actin (myoepithelial markers) as well as CK6+ (a putative progenitor marker) suggesting that this tumor is indeed basal and is of a mixed lineage (Appendix 7). Finally, expression profiling specific for the transgene confirms that the tumor is indeed positive for the Crk transgene (Appendix 6). These findings are important, as basal tumors in human breast cancer patients are often associated with poor prognosis (25). Further immunohistochemistry on the MMTV-CrkII adenosquamous carcinoma will be done to ensure that the tumors are indeed CK8 positive and ErbB2,

ER, and PR negative, similar to human patients and the molecular mechanisms by which CrkII causes tumor development is ongoing via Western blot analysis.

Immunohistochemical staining of CrkII in both normal mammary epithelium and tumor epithelium is both nuclear and cytoplasmic. However, little is known about the role of CrkII in the nucleus. To confirm the staining is indeed specific, another antibody specific for CrkII is being tested. Furthermore, cytoplasmic and nuclear extracts will be blotted for CrkII. Co-immunoprecipitation of CrkII from nuclear extracts will also be performed to identify potential binding partners of CrkII.

As previously mentioned, a division of our lab, the Breast Cancer Functional Genomics Group (BCFGG), have generated gene expression profiles from laser capture microdissected tumor and matched-normal epithelium from human breast cancer patients. For each patient whose tumor has been analyzed by microarray analysis, a 10-year follow-up history has been documented in a database developed by our lab. Greg Finak, another PhD student in our lab, has identified that CrkL (which is 60% homologous to CrkII) is highly expressed in basal tumors compared to other tumor types (Appendix 8). We are currently examining whether CrkII is highly expressed in this gene signature as well. Furthermore, I will be carrying out immunohistochemical staining for CrkII on human tissue arrays to examine whether CrkII is over-expressed in basal versus luminal breast cancers.

In each line studied, abnormal mammary development was also found. For instance, five to twelve week old virgin mice were found to have delayed ductal outgrowth, compared to their negative FVB littermates (Appendix 9). In these mice at 12 weeks of age, terminal end buds are persistant and disorganized, and have not yet reached the edges of the fat pad, whereas in their wild-type litter mates, these buds are regressed and the fat pads are filled with branching ducts. This phenotype is similar to other mouse models, such as MMTV-EphB4, MMTV-Cripto and MMTV-Krct (26, 27, 28). Although many other mouse models which show this phenotype do not examine how delayed outgrowth occurs, I have shown that it appears to be the result of increased collagen surrounding the terminal end buds. This was determined by Trichrome staining and indicates a slowed development of the mammary gland (Appendix 10). However, whether CrkII induces the formation of collagen or inhibits its breakdown remains to be determined. Any potential changes in cellular proliferation, apoptosis, and differentiation are currently being examined through immunohistochemistry.

In other mice of these lines (at 20 weeks and 1 year of age), there is increased ductal branching, suggesting enhanced proliferation of the epithelial branches and this is accompanied by increased PCNA staining and an increased association with p130Cas (Appendix 11). MMTV-p130Cas mice show a similar branching phenotype (22). Western blot analysis did not reveal any significant changes in the Akt, Erk or JNK pathways, however it is possible that any changes in the activation of these pathways may be missed at this particular time point (20 weeks) (Appendix 12). In addition to tumor development, hyperplasia and precocious lobuloaveolar development has also been found in aging virgin mice and further work needs to be done to characterize whether these phenotypes are due to the transgene by staining for Crk (Appendix 13). Lactation in these mice appear normal.

Future work with MMTV-CrkII line includes mating these animals ErbB2 transgenic mice (NDL2-5) to determine if a decrease in latency or an increase in metastasis is achieved (29). At the present time, the addition of CrkII does not alter ErbB2 tumor latency or pathology (Appendix 14). Both Crk and ErbB2 transgenes are expressed in the tumors. Enhanced metastasis will be achieved by injecting cell lines derived from ErbB2 or CrkII-ErbB2 tumors into the mammary fat pad or tail vein of FVB mice.

In addition to the crosses with ErbB2 mice, the effects of high CrkII expression are currently being studied in mice undergoing either pregnancy or involution. Thus, the effects of CrkII expression on all aspects of mammary gland development will be characterized by immunohistochemistry, and if feasible, Western blot analysis. Finally, this data will be compared to endogenous CrkII levels, as CrkII has not been examined in the mammary gland. I hope to finish all aspects of this project by the end of the year and submit these data for publication.

The MMTV-CrkI mice have been generated and 13 founder lines were established. Of these lines, 4 were terminated as the mice were not breeding properly. Expression profiling is currently underway to

determine lines which show high levels of CrkI expression. In contrast to the CrkII mice, 12/16 of CrkI animals at 12 weeks of age have normal ductal outgrowth, whereas only 4/16 showed delayed ductal outgrowth which is similar to CrkII mice (Appendix 15). A potential lactational defect in these mice is currently being examined as there are patches of the mammary gland which do not appear to be producing milk. Lactation in CrkII mice were normal, suggesting a key difference between these two Crk isoforms. Although their expression profiles should be compared to the levels of expression of CrkII mice, this suggests that both CrkI and CrkII proteins have variable roles in mammary gland development. Interestingly, CrkI staining via immunohistochemistry is localized at cell-cell junctions, unlike CrkII which is mainly nuclear (Appendix 15).

Future work of the MMTV-CrkI line is to decrease the number of founder lines from 13 to 2 and then to set up multiparous and aging virgin cohorts. The role of CrkI in all aspects of mammary gland development (puberty, pregnancy, lactation and involution) will also be examined. These mice may be mated with ErbB2 animals to also determine if a decrease in tumor latency or an increase in metastatic potential can be achieved. Thus, in the upcoming year, I hope to set up all the animals I need to examine the role of CrkI in mammary gland development as well as tumor development.

Lastly, the MMTV-CrkL construct has been made. This construct has not been sent to the transgene facililty for several reasons. Firstly, there is not enough room in the animal facility at the present time. Secondly, a serine to threonine mutation was discovered in the original cDNA obtained from Invitrogen. Through site-directed mutagenesis, this correction was made and at the present time, the construct has been isolated and is ready to be sent for injection. Since MMTV-CrkL has a V5 tag, studies were performed to ensure the tagged protein acts in an identical manner to the native protein. At the present time, the tagged protein has been shown to interact with the endogenous CrkL binding partner, Gab1, which is enhanced upon HGF stimulation (Appendix 16). One final experiment, showing that the CrkLV5 tag can promote Rac activation is being carried out. If I can confirm that the tagged protein acts similar to the endogenous CrkL protein, then I will feel confident that the construct is ready to send out. In the upcoming year, I hope to examine virgin and lactating mice from these lines to determine expression levels once these mice are generated.

KEY RESEARCH ACCOMPLISHMENTS

* Crk proteins are important regulators of breast cancer migration and invasion

* High CrkI/II levels affect mammary gland development

* Over-expression of CrkII can lead to breast cancer development

* Tumors derived from CrkII may be derived from epithelial progenitor cells or through EMT

* Over-expression of Crk proteins in human breast cancer may be associated with poor prognosis disease

REPORTABLE OUTCOMES

Manuscripts

Rodrigues, SP^, Fathers, KE^, Chan, G, Zuo, D, Halwani, F, Meterissian, S, Park, M. 2005. CrkI/II function as key signalling nodes for migration and invasion of cancer cells. Molecular Cancer Research. 3 (4):183-94
^ - Both authors contributed equally to this work.

Abstracts

Rodrigues, SP, Fathers, KE, Chan, G, Park, M. 2004. Crk adaptor proteins play an essential role in cellular migration and invasion in multiple human cancer cell lines. Twentieth Annual Meeting on Oncogenes. Frederick, USA

Fathers, KE, Rodrigues, SP, Park, M. Crk adaptor proteins play a key role in cellular migration and invasion in human cancer. 2005. American Society of Cellular Biology - Systems Integration in Directed Cell Motility. Seattle, USA

Fathers, KE, Rodrigues, S, Park, M. 2006. Crk adaptor proteins play a key role in cellular migration and invasion in human cancer. Canadian Breast Cancer Research Alliance - Reasons for Hope. Fourth Scientific Conference. Montreal, Canada

Fathers, KE, Monast, A, Rodrigues, SP, Park, M. 2006. MMTV-CrkII mice display a defective ductal outgrowth phenotype in peripubertal mice. Gordon Research Conference - Mammary Gland Biology. Barga Italy.

Presentations

Fathers, KE. Crk adaptor proteins act as key signaling integrators in human breast cancer. Molecular Oncology Group Seminar Series. McGill University, Montreal, Canada.

Animal Models

MMTV-CrkII
MMTV-CrkI

Cell Lines

MDA-231 breast cancer cell lines stably expressing shRNA targeting CrkI/II
MDA-231 breast cancer cell lines stably expressing shRNA targeting CrkL
MMTV-CrkII tumor cell lines, derived from two MMTV-CrkII tumors
MMTV-CrkII/ErbB2 tumor cell lines, derived from ErbB2/CrkII + tumors

CONCLUSION

The present study demonstrates that Crk adaptor proteins play an important role in integrating signals for migration and invasion of highly malignant breast cancer cell lines. This has important implications as elevated levels of Crk are observed in various human cancers, such as breast. Hence, our findings demonstrating a role for Crk as a key integrator of signals for cell migration and invasion identifies a potential role for Crk in metastatic progression. Future work from this project will show the importance of Crk adaptor proteins during metastasis, which is the rate-limiting factor in cancer treatment. Furthermore, this project suggests that over-expression of Crk in a progenitor cell population of the mammary gland may aid in the development of “basal” tumors, which are associated with poor prognosis in human patients. In addition to targeting a progenitor population, Crk tumors may also give rise to EMT-type tumors, which are thought to be highly invasive. Thus, our study on Crk adaptor proteins has the potential to identify some of the molecular events, which can occur during breast cancer metastasis and tumorigenesis. This may provide information, which could be used to develop effective anti-metastatic treatments for breast cancer.

“SO WHAT”

Metastasis is a major cause of morbidity and mortality in human malignancies, and is the driving force behind the incessant pursuit of “anti-metastatic” and adjuvant therapies. For instance, patients with metastatic breast cancer have a median survival of only 2 to 3 years and twenty percent of the patients who present with bone metastases have only a five year survival from the time of diagnosis (30). As a result, current therapies for metastatic breast cancer are aimed at improving palliative care rather than complete remission (30). Further progress in this field of research may be achieved through a better understanding of the various molecular processes defining the complexity and multi-step nature of tumor cell dissemination, otherwise known as the metastatic cascade. My research has shown that Crk adaptor proteins are key integrators for cellular migration and invasion in various breast cancer cell lines, regardless of their inherent

mutations and upstream signals. This suggests that Crk adaptor proteins may be viable targets for the metastasis, as cellular migration and invasion are key compontents of the metastatic cascade. Through my future work using in vivo metastatic mouse models as well as microarray analysis, I may be able to dissect signaling pathways responsible for metastasis. This will be an important step towards developing targeted therapeutics for breast cancer metastases.

In addition to identifying a novel role for Crk adaptor proteins in breast cancer metastasis, I have shown that CrkII may be important in the promotion of breast cancer. Preliminary results show that over-expression of CrkII may lead to the formation of EMT and "basal"-like tumors, which are associated with decreased survival in human patients. As these "basal" breast tumors are triple negative (Her2, ER, PR negative); treatment options for these tumors are limited. Thus, the need for identifying potential targets for these tumors is important. Through gene expression profiling, our lab has shown that CrkL (which shares 60% homology to CrkII) is over-expressed in human basal tumors. Future work will examine whether Crk adaptor proteins are over-expressed in basal vs. luminal tumors through tissue arrays and the development of MMTV-CrkI and MMTV-CrkL mouse models will illustrate whether these proteins also drive a basal tumor phenotype. Thus, the data generated from this study has the potential to identify Crk adaptor proteins as valuable targets for these types of breast tumors.

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CrkI and CrkII Function as Key Signaling Integrators for Migration and Invasion of Cancer Cells

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Abstract

Crk adaptor proteins play an important role during cellular signaling by mediating the formation of protein complexes. Increased levels of Crk proteins are observed in several human cancers and overexpression of Crk in epithelial cell cultures promotes enhanced cell dispersal and invasion, implicating Crk as a regulator of invasive responses. To determine the requirement of Crk for invasive signals, we targeted the *CRKI/II* gene by RNA interference. Consistent knockdown of CrkI/II was observed with two small interfering RNA targeting sequences in all human cancer cell lines tested. CrkI/II knockdown resulted in a significant decrease in migration and invasion of multiple malignant breast and other human cancer cell lines (MDA-231, MDA-435s, H1299, KB, and HeLa). Moreover, CrkI/II knockdown decreased cell spreading on extracellular matrix and led to a decrease in actin stress fibers and the formation of mature focal adhesions. Using immunohistochemistry, we show elevated CrkI/II protein levels in patients with breast adenocarcinoma. Together, these studies identify Crk adaptor proteins as critical integrators of upstream signals for cell invasion and migration in human cancer cell lines and support a role for Crk in metastatic spread. (Mol Cancer Res 2005;3(4):183–94)

Introduction

The progression of cancer to metastasis is dependent, in part, on the deregulation of signaling pathways involved in migration and invasion. In response to the extracellular environment, epithelial sheets or individual cells respond to different migratory signaling pathways to invade surrounding tissue (1). These transitions are also prominent in normal physiologic processes such as embryonic development, wound healing, and organogenesis. The dissection of these signaling pathways will be an important step towards developing targeted therapeutics for metastases.

Hepatocyte growth factor (HGF) and its receptor tyrosine kinase, Met, are potent modulators of epithelial dispersal and invasion, *in vitro* and *in vivo* (2). Although many signaling pathways have been identified to coordinate this process (3), we have identified a role for Crk adapter proteins downstream from the HGF/Met receptor tyrosine kinase, in both the dispersal of epithelial colonies and in epithelial morphogenesis induced by HGF (4-7).

Crk was originally isolated as the oncogene fusion product of the CT10 chicken retrovirus (v-Crk; ref. 8). Cellular homologues of v-Crk include the *c-CRK* gene, which encodes two alternatively spliced mRNAs that give rise to two proteins (c-CrkI and c-CrkII), and a second gene, *c-CRKL*. Crk proteins are composed of one Src homology 2 (SH2) and, one or two Src homology 3 (SH3) domains (9). They are adaptor proteins that direct the assembly of multiprotein signaling complexes. Crk adaptor proteins and their effectors are highly conserved throughout evolution. The Crk SH2 domain binds a specific phosphorylated tyrosine motif found in proteins involved in cell spreading, actin reorganization, and cell migration. These Crk-SH2 binding proteins include the focal adhesion components, p130Cas and paxillin (10), growth factor receptor tyrosine kinases, and a docking protein Gab1, involved in epithelial dispersal and morphogenesis (5, 11, 12). The NH_2-terminal SH3 domain of CrkII interacts constitutively with proline-rich motifs present within several proteins, including C3G, a nucleotide exchange factor for Rap1 (13), DOCK180, an exchange factor for Rac1 (14, 15), as well as the Abl tyrosine kinase (16), tyrosine phosphatase (protein tyrosine phosphatase 1B; ref. 17), the p85 subunit of phosphatidylinositol 3-kinase (18), and the c-Jun-NH_2-kinase (1). Binding proteins for the COOH-terminal SH3 domain are still poorly understood.

Genetic studies in *C. elegans* have shown a role for CrkII and DOCK180 in phagocytosis and polarized cell migration (19, 20). Similarly, the overexpression of CrkII or CrkL in mammalian cells *in vitro* enhances migration when assayed as single cells (21-25) and promotes the dispersal of epithelial cell colonies (6). CrkII has been identified as a mediator of cell migration through its association with p130Cas and paxillin (23) as well as the Rac exchange

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factor DOCK180 (14). Through these interactions, a role for Crk proteins has been proposed in the regulation of cell migration, morphogenesis, invasion, phagocytosis, and survival (10).

Aggressive and invasive tumors can aberrantly activate intracellular pathways required for cell migration and invasion. Elevated levels of CrkI and CrkII proteins and mRNA are found in various human tumors (26), including glioblastomas (27) and lung cancers (26, 28). Moreover, c-CrkI/II mRNA expression was predominantly increased in lung tumors that were at more advanced stages as well as those associated with poor survival (26). Together, these studies support a role for Crk in tumor progression.

Much of our understanding about the role of Crk in mammalian cells derives from overexpression studies. Although RNA interference of CrkII has been shown to limit membrane ruffling of aortic endothelial cells (29) and CrkII (30) and CrkL knockout mice (31) have been established, limited analysis of cells derived from these mice has been published to date. To investigate a requirement for Crk in migration and invasion in human tumors, we have examined the association of Crk protein levels in human breast cancer and have used RNA interference directed against CrkI/II in human cancer cell lines. We show elevated levels of CrkI/II proteins in breast adenocarcinoma and show a key role for Crk adaptor proteins in the enhanced migration and invasion signaling programs of multiple human cancer cell lines.

Results

CrkI/II Proteins in Human Breast Cancer

The molecular mechanisms important to the progression of breast cancer invasion and metastasis are poorly understood. The most important prognostic indicator in breast cancer is the lymph node status. We examined the level of proteins from the *c-CRK* gene, CrkI and CrkII, by immunohistochemistry in primary breast tumors using two cohorts of patients with breast adenocarcinoma, including 8 node-negative stage I cancer patients in group 1, and 12 node-positive stage III cancer patients in the other. The patient characteristics are summarized in Table 1. Immunohistochemical analysis of paraffin sections, using sera that recognized CrkI and CrkII, revealed positive staining for CrkI/II protein in adjacent normal ductal epithelial cells as well as in adenocarcinoma cells. Using a semiquantitative analysis, 60% of all the tumors displayed elevated immunostaining for CrkI/II in comparison with adjacent normal ducts (Fig. 1A). By cohort, the elevated CrkI/II immunostaining was evident in 63% of stage I and 58% of stage III samples. There was no statistical difference in proportion of tumors displaying elevated CrkI/II levels between stages (χ^2 test, $P = 0.853$).

CrkI/II Protein Levels Are Decreased in Multiple Human Cancer Cell Lines following Small Interfering RNA Treatment

To investigate the function of Crk adaptor proteins in migration and invasion of human cancer cell lines, we used the previously published CrkI/II small interfering RNA (siRNA) duplex targeted to the human CrkI and CrkII mRNA, siCrk1, with the sequence 5′-AAUAGGAGAUCAAGAGUUUGA-3′ and 5′-UCAAACUCUUGAUCUCCUAUU-3′ (29) along with a novel, secondary siRNA, termed siCrk2, with the sequence 5′-AGGAGACAUCUUGAGAAUCdTdT-3′ and 5′-GAUUCUCAAGAUGUCUCCUdTdT-3′ (Fig. 2A). The ability of siCrk to knock down CrkII protein levels was tested by cotransfecting the human cervical carcinoma cells HeLa with cDNA expressing human CrkII. In the absence of siRNA, CrkII was robustly expressed following transient transfection with a CrkII cDNA expression vector for 24 hours and this was reduced in the presence of siCrk1 (Fig. 2B). However, it should be noted that this reduction was not to endogenous levels, suggesting that the siCrk1 may not be sufficient to completely ablate the high levels of expression from the CrkII cDNA plasmid. In HeLa cells, endogenous CrkII protein is readily detectable whereas CrkI is only expressed at low to nondetectable levels. To examine the ability of siRNAs to knock down endogenous Crk protein levels, HeLa cells were transfected with increasing concentrations of siCrk1, with a threshold of 100 pmol/L (Fig. 2C). CrkII protein levels, 24 hours posttranfection, were efficiently suppressed in a dose-dependent manner (Fig. 2C). Under all experimental conditions, levels of growth factor receptor binding protein 2 (Grb2) in cell lysates remained similar, indicating that overall protein levels were not decreased by siCrk1 treatment (Fig. 2C). Transfection with a control nontargeting siRNA did not reduce CrkII protein levels (Fig. 2D). The decrease in CrkII protein levels was observed by Western blot analysis to last up to 5 days (120 hours) posttransfection (Fig 2E). Optimal conditions for transfection with siRNA were established using HeLa cells and were subsequently applied to multiple human cancer cell lines including MDA-231, MCF-7, and MDA-435s breast cancer cell lines, KB oral epithelial cancer cells, and H1299 lung cancer cells. All cell lines examined express CrkI and CrkII and although the levels of CrkI and CrkII vary in response to siCrk1, suppression of Crk was observed in all cancer cell types examined (Fig. 2F).

We examined the specificity of siCrk1 and siCrk2 in MDA-231 cells as both siRNAs are targeted to different regions of the *CRK* gene (Fig 2A). Endogenous CrkI and CrkII are readily detected by Western blot analysis of total proteins in MDA-231 cells, and protein levels for both were decreased following transient transfection with either siCrk1

Table 1. Patient Cohorts Used to Examine CrkI/II Protein Expression Levels by Immunohistochemistry

	Stage I	Stage III
No. patients	8	12
Median age (y)	62	64
No. ductal adenocarcinoma	8	8
No. lobular adenocarcinoma	0	4
% 5-y survival	87.5	50
Median overall survival (d)	3,360	1,733
No. recurrences	2	5
Median disease-free survival (d)	3,360	905
No. with elevated Crk protein level*	5	7

*χ^2 test, $P = 0.853$.

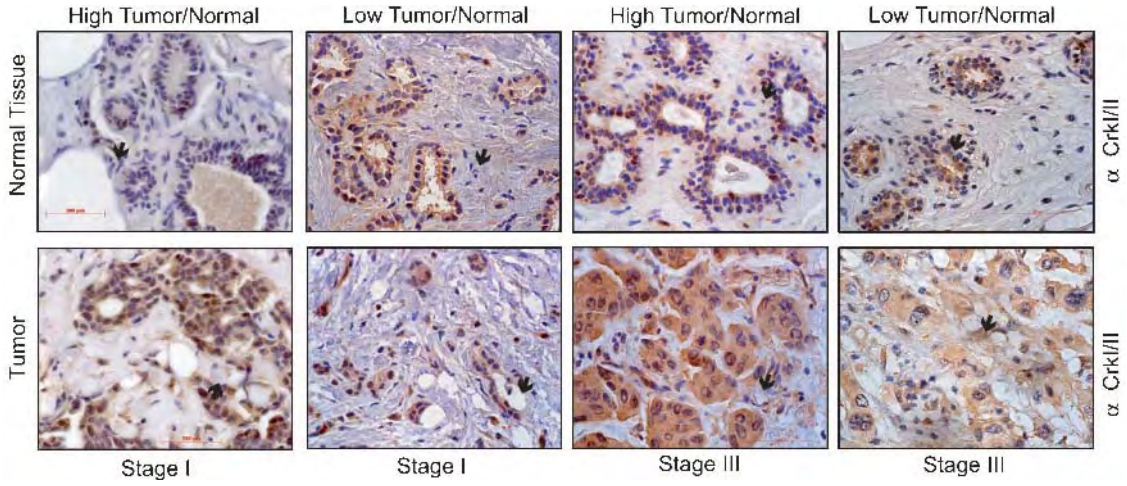

FIGURE 1. Immunohistochemistry analysis of CrkI/II proteins in stage I and III breast cancers. Paraffin sections of tumor specimens were analyzed by immunohistochemistry using an anti-Crk monoclonal antibody that recognizes both CrkI and CrkII (*black arrows*) and counterstained with hematoxylin. Digital photographs were captured using a 40× objective.

or siCrk2 (Fig. 3A), however, complete ablation of detectable CrkI or CrkII protein was not observed. To assess the effect of transfection efficiency and the cellular uptake of siRNA, Cy3-labeled siRNAs were transiently transfected into MDA-231 breast cancer cells. As shown in Fig. 3B, nearly 100% of MDA-231 cells are positive following transient transfection with Cy3-labeled siRNAs. To assess whether suppression of Crk protein levels had an effect on apoptosis or impaired cell proliferation during the time course of our biological assays, cell proliferation rates were analyzed over a 72-hour period in MDA-231 mock cells and siCrk-transfected cells, during which time CrkI/II protein levels remain suppressed. During that period, no difference in cell number was observed between the two cell types (Fig. 3C). The induction of early apoptotic events was assayed by Annexin V staining in both HeLa and MDA-231 cells. Forty-eight hours posttransfection, control cells transfected with lipid alone and siCrk1-transfected cells were incubated for 15 minutes with 100 μL of Annexin V-fluorescein. As a positive control for apoptosis, cells were exposed to 50 J/m^2 UV light and incubated at 37°C overnight, and assayed in parallel with siRNA-transfected cells. Annexin V staining confirmed that both HeLa and MDA-231 cells underwent activation of apoptotic events when exposed to 50 J/m^2 UV light. However, no significant activation of early apoptotic events was observed in either mock transfected cells or siCrk-transfected cells (Fig. 3D). These results show that the decrease in Crk protein levels observed following transfection with siCrk does not promote apoptosis or alter proliferation of MDA-231 cells maintained in serum.

Migration of Multiple Cancer Cell Lines Is Decreased following CrkI/II Small Interfering RNA Treatment

Previous studies have shown that overexpression of Crk enhances cell motility and promotes epithelial dispersal. However, the overexpression of mutant Crk proteins acting as dominant interfering proteins decreased these biological responses, implicating Crk in the transmission of integrin-dependent or growth factor–dependent signals for cell migration (4-6, 32-35). To assess whether Crk adaptor proteins act as critical integrators of signals for cell migration in human cancer cell lines, siRNA duplexes targeting CrkI/II, as described above, were transfected into HeLa, MDA-231, MDA-435s, KB, and H1299 cells and their effect on cell migration and invasion was assayed. The inherent migration capacity of these cell lines in serum was assayed 48 hours following transfection with siCrk using transwell migration assays as previously described (23, 36). The degree of "knockdown" of the *CRK* gene products, CrkI and CrkII, was assayed for each experiment where half of transfected cells were processed for biochemistry. For each cancer cell line tested, a significant decrease in cell migration was observed after 24 hours (Fig. 4A). For example, migration of breast cancer cell lines, MDA-231 and MDA-435s, was routinely decreased by 75% following transfection with siCrk. A similar decrease in migration of 50% was observed for the H1299 and KB cell lines, whereas the migration of HeLa cells transfected with siCrk was consistently reduced by 30% when compared with control transfected cells or HeLa cells transfected with a nontargeting siRNA (Fig. 4A). All experiments were done at least thrice per cell line, and all showed comparable results with $P < 0.05$. For all cell lines tested, a decrease in CrkII and CrkI protein levels was observed (Fig. 4B).

Overexpression of Crk enhances epidermal growth factor–induced cell dispersal and tubulogenesis (5), and dominant negative mutants of Crk block HGF-induced cell scatter in Madin-Darby canine kidney cells (6). To determine if Crk is required for HGF-enhanced cell migration in aggressive cancer cells, we treated MDA-231 and HeLa cells with siCrk1 or siCrk2 in the presence or absence of HGF (34 ng/mL). HGF stimulation enhanced migration of MDA-231 cells by ~50% when compared with their unstimulated counterparts (Fig. 4C). In a similar manner to unstimulated MDA-231 cells, Crk

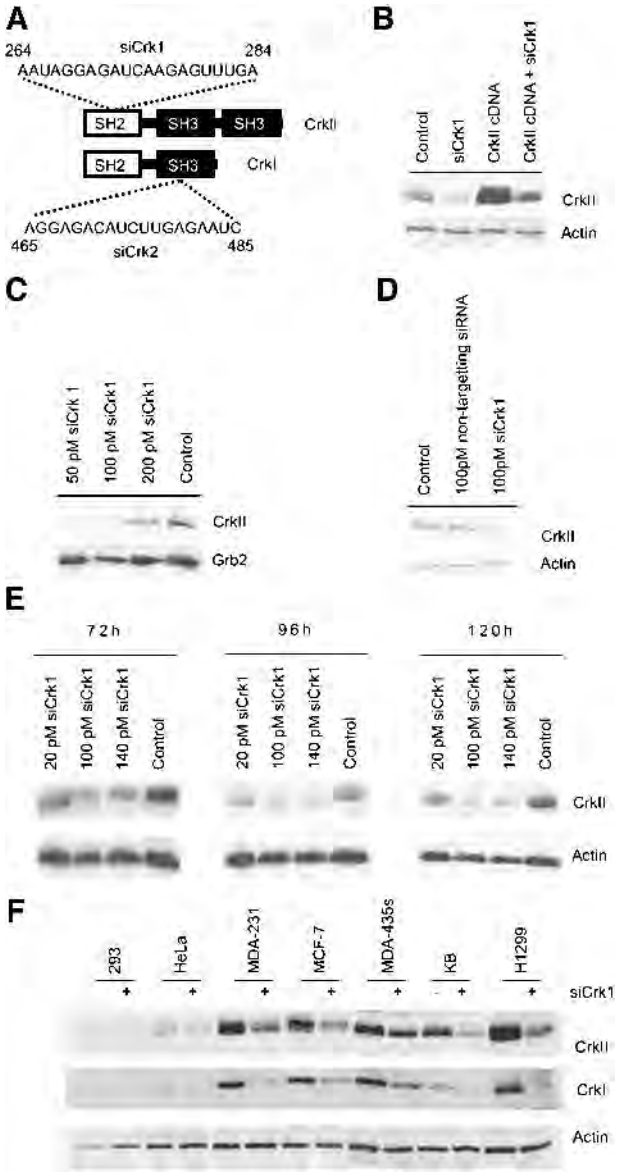

FIGURE 2. Targeting and design of CrkI/II specific siRNAs in HeLa cells. **A.** CrkI/II option 1 and 2 siRNA constructs. **B.** Western blot analysis of whole cell lysates (HeLa) with an anti-CrkI/II or anti-actin sera revealed that the Crk siRNA duplexes efficiently and specifically target its respective RNA, even when cotransfected with a CrkII-expressing plasmid. **C.** HeLa cells were transfected with CrkI/II at various concentrations and protein lysates were analyzed by Western blot using antibodies against CrkI/II. Growth factor receptor binding protein 2 (*Grb2*) levels were used as a loading control. **D.** HeLa cells were transfected with a siRNA unable to be targeted by the RISC complex or CrkI/II targeting siRNAs to assess specificity of the siRNA procedure. Levels of Crk adaptor proteins were obtained by Western blot analysis. Actin protein was used as a loading and specificity control. **E.** HeLa cells were transfected with siCrk1 at various concentrations as previously described. A time course was established by lysing the cells 72, 96, and 120 hours posttransfection. CrkI and CrkII protein levels were analyzed by Western blot using antibodies against CrkI/II. Actin protein levels were used as a loading control. **F.** A panel of human cancer cell lines consisting of HEK293, HeLa, MCF-7, MDA-231, MDA-435s, KB, and H1299 cells were transfected with CrkI/II siRNA and protein lysates were analyzed by Western blot for CrkI/II protein levels. Actin protein levels were used as loading control.

siRNA pretreatment decreased HGF induced migration by 60% (Fig. 4C). Similar results were obtained with HeLa cells (data not shown). These results support a role for Crk adaptor proteins as central integrators for upstream signals involved in cell migration. These results also highlight a possible role for Crk proteins in cell invasion.

Crk Small Interfering RNA Inhibits Invasion of Multiple Cancer Cell Lines

To assay the effect of Crk knockdown on the ability of cancer cells to invade an extracellular matrix, the invasion of cancer cells transfected with siCrk in Fig. 4A was examined using transwells coated with 100 $\mu g/cm^2$ of matrigel. Forty-eight hours posttransfection, 5×10^4 cells/mL were seeded on top of the matrigel coating and incubated for 24 hours. Cells that invade through the matrix are visualized by staining the lower membrane with 0.1% crystal violet in 20% methanol and quantified. The ability of MDA-231, MDA-435s, H1299, and KB cells to invade through matrigel was decreased up to 80% compared with control cells when transfected with siCrk, whereas that of HeLa cells was decreased by 60% (Fig. 5A). All experiments were done at least thrice per cell line, where all showed comparable results with $P < 0.05$. In all cases, transfection with the control siRNA gave no significant decrease in cell invasion nor a decrease in CrkII protein levels (Fig. 5B). Hence, depletion of CrkI/II proteins by siCrk decreased cell invasion through matrigel in a similar manner to cell migration. To further assess the selectivity of action of Crk siRNA, we examined human breast cancer T47D cells that show increased invasion following overexpression of CrkII (Fig. 5B; ref. 6). Control T47D cells show a decrease in cell invasion following transfection with siCrk1 (28%, Fig. 5C); moreover, the enhanced cell invasion of CrkII overexpressing cells was reversed following transfection with siCrk1 (Fig. 5C).

CrkI/II Small Interfering RNA Decreases Cell Adhesion

Cell migration requires the ability to form and break focal adhesion complexes, a process that occurs during cell adhesion and spreading. To examine the effect of siCrk1 on cell adhesion and spreading, MDA-231 cells were trypsinized and replated on fibronectin 48 hours posttransfection and subjected to videomicroscopy. Time lapse videomicroscopy of mock and siCrk-transfected MDA-231 cells revealed that mock control cells begin to spread and develop lamellipodia by 10 minutes post-plating and by 30 minutes have developed large lamellipodia (Fig. 6A). In contrast, siRNA-transfected cells display smaller lamellipodia and cells retain a round and refractile morphology for a prolonged time, when compared with control cells, consistent with decreased spreading. Moreover, when assayed for attachment and spreading at later times, cells transfected with siCrk formed less stable adhesions and detached from the substratum (Fig. 6B and C).

To examine the consequence of CrkI/II knockdown on the actin cytoskeleton and focal adhesions, we examined the localization of focal adhesion targeting proteins paxillin and vinculin by indirect immunofluorescence. MDA-231 cells transfected with siCrk1 were trypsinized and replated onto

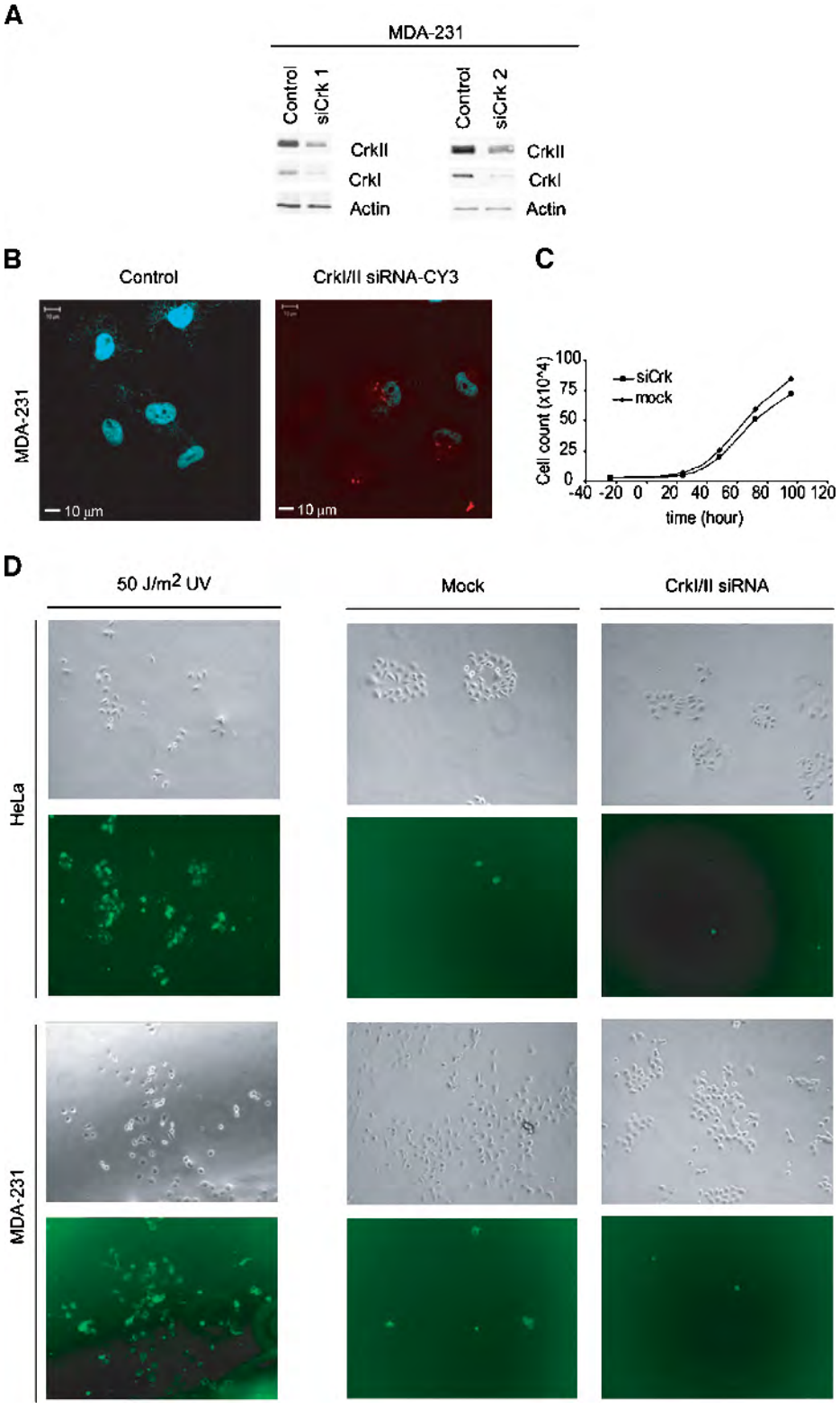

FIGURE 3. CrkI/II targeting siRNAs specifically knock down protein levels in a panel of tumor cell lines. **A.** MDA-231 cells were transfected with CrkI/II and protein lysates were analyzed by Western blot using antibodies against CrkI/II. Actin protein levels were used as a loading control. An additional construct of CrkI/II siRNA was designed and its protein knockdown efficiency was evaluated by Western blot analysis. Actin protein levels were used as a loading control. **B.** CrkI/II siRNAs were labeled with a CY3 marker and transfection efficiency was assessed by indirect immunofluorescence. All cells show CY3 staining, indicating that CrkI/II siRNAs were readily transfected into MDA-231 cells. Images were acquired using a Zeiss confocal microscope and a 63× objective. **C.** Transfection of CrkI/II siRNAs does not affect cell proliferation. In MDA-231 cells transfected with lipid alone and CrkI/II siRNAs, cell proliferation rates were analyzed. Cell counts were taken every 20 hours. No significant change in cell proliferation was observed posttransfection with CrkI/II siRNAs. **D.** Transfection of CrkI/II siRNA does not affect cell death. HeLa and MDA-231 cells were transfected as previously described. Positive controls were exposed to 50 J/m^2 of UV radiation and incubated overnight. Cells were analyzed 48 hours posttransfection for early-apoptotic stages using Annexin V staining and for necrosis using propidium iodide (data not shown). Both assays showed that the transfection with siRNAs did not induce cell death. Images were obtained using a 10× objective on a Zeiss AxioVert 135 microscope.

fibronectin-coated coverslips 48 hours posttransfection. When fixed 16 hours post-plating, control MDA-231 cells had developed abundant actin stress fibers, as visualized by phalloidin, whereas siCrk1-transfected cells showed reduced actin stress fibers throughout the cell. Bundled actin was still observed within lamellipodium-like structures at the cell periphery. Consistent with a decrease in actin stress fibers, siCrk1-transfected cells show reduced focal adhesions as

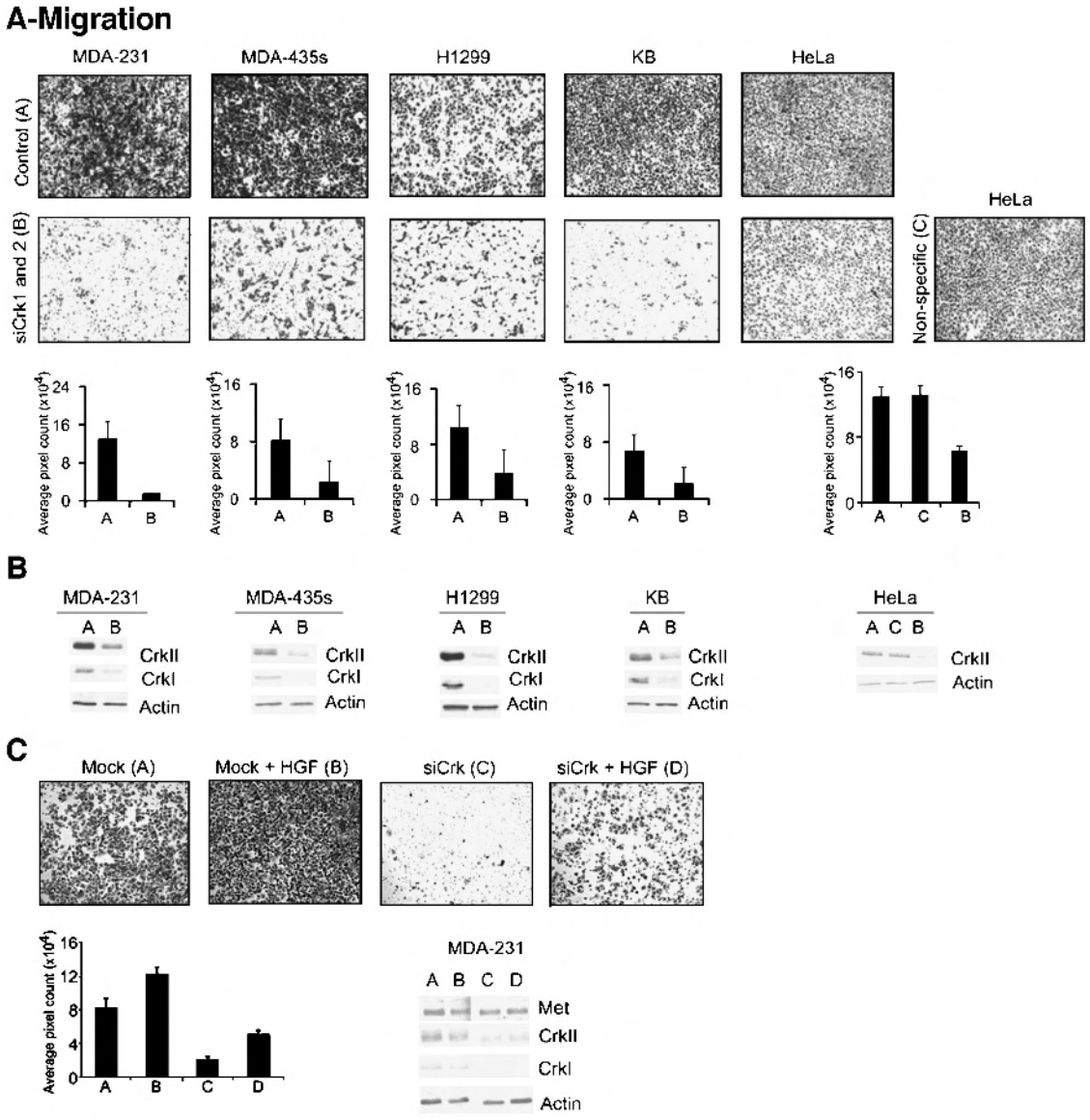

FIGURE 4. A decrease in Crk adaptor protein expression leads to decreased cell migration in multiple cancer cell lines. **A.** MDA-231, MDA-435s, KB, and H1299 cells transfected with siCrk1 or siCrk2 were analyzed for their migration capacity in the presence of serum 48 hours posttransfection. Similarly, HeLa cells transfected with CrkI/II or a nontargeting siRNA were seeded into modified boyden chambers and assayed. Cells remaining on the bottom of the porous membrane were fixed in formalin phosphate, stained with 0.2% crystal violet, extensively washed with H_2O, and left to dry overnight. Using a Retiga 1300 digital camera and a Zeiss AxioVert 135 microscope, bottom layers of the transwell were imaged in five separate fields for each condition using a 10× objective in phase contrast. Image analysis of these assays was carried out using Northern Eclipse and Scion Image. A minimum of three experiments was done. Bars, SD of the three experiments. **B.** For each experiment, the extent of CrkI/II knockdown was assessed by Western blot analysis. Cells were trypsinized 48 hours posttransfection and either seeded into boyden chambers for migration or lysed to estimate CrkI/II protein levels. Proteins derived from whole cell lysates were separated by PAGE, transferred to a membrane, and immunoblotted with anti-CrkI/II sera or actin. **C.** MDA-231 cells transfected with siCrk1 or siCrk2 were analyzed to examine cellular migration in the presence or absence of HGF (34 ng/mL) as described above. A minimum of three experiments was done. Bars, SE of the three experiments.

observed through reduced vinculin and paxillin staining when compared with control cells (Fig. 7). Together, these results support a role for Crk proteins for the establishment or maintenance of strong cell-matrix adhesions.

Discussion

Despite the accumulation of evidence for a function of Crk adaptor proteins in cell adhesion and cell migration, its significance in inherently motile cancer cells still remains elusive. Much of what has been proposed about CrkI/II in epithelial cells is based on overexpression studies or the use of dominant interfering mutants of CrkI/II. These approaches, although informative, are limited by the challenges inherent in overexpression systems. In this article, we report that knockdown of CrkI/II expression by RNA interference leads to a dramatic decrease in cell migration and invasion in multiple human tumor cell lines. Duplex siRNA–based

methods have the advantage of being able to nearly abrogate RNA expression and proteins of CrkI and CrkII and permit the consequences to be examined in multiple tumor cell lines (Fig. 2). Moreover, the acute and transient effects of siRNA-mediated CrkI/II down-regulation provide an alternative experimental approach to study the functions of CrkI and CrkII, avoiding the possible effect of compensatory mechanisms developed in knockout mice.

The present study establishes that transfection with CrkI/II siRNA duplexes attenuates CrkI and CrkII protein expression levels (Figs. 2 and 3), without affecting the expression of endogenous proteins, such as actin or another adaptor protein, Grb2, demonstrating the specificity of this gene targeting approach. Furthermore, the transfection with an siRNA duplex designed against a separate region of the *CRKI/II* gene was similarly effective at specifically knocking down CrkI/II protein

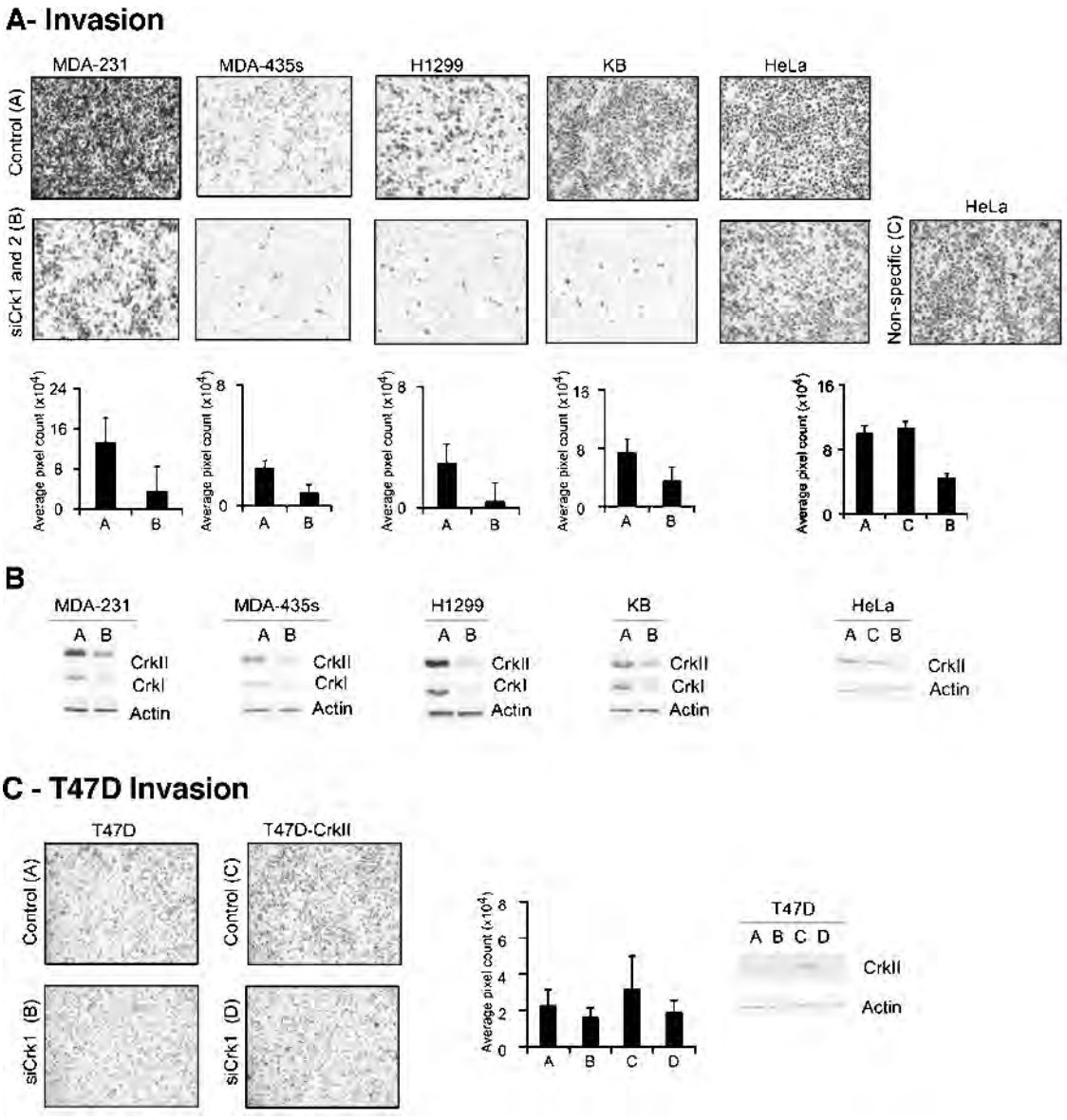

FIGURE 5. Crk siRNA inhibits invasion of cancer cells. **A.** Assays for cell invasion were carried out 48 hours posttransfection using 5×10^4 cells. Cells were seeded onto matrigel covered boyden chambers and assayed. Cells remaining on bottom of the porous membrane were fixed in formalin phosphate, stained with 0.2% crystal violet, extensively washed with H_2O, and left to dry overnight. Using a Retiga 1300 digital camera and a Zeiss AxioVert 135 microscope, bottom layers of the transwell were imaged in five separate fields for each condition using a 10× objective in phase contrast. Image analysis of these assays was carried out using Northern Eclipse and Scion Image. A minimum of three experiments was done. Bars, SD of the three experiments. **B.** For each experiment, the extent of CrkI/II knockdown was assessed by Western blot analysis. Cells were trypsinized 48 hours posttransfection and either seeded into boyden chambers for invasion or lysed to estimate CrkI/II protein levels. Proteins derived from whole cell lysates were separated by PAGE, transferred to a membrane, and immunoblotted with anti-CrkI/II sera or actin. **C.** To confirm the specificity of siCrk1 within a biological context, we examined the invasion capacity of T47D cancer cells and those that overexpress CrkII compared with their siCrk1-transfected counterparts, using the assay described above. A minimum of three experiments was done. Bars, SD of the three experiments.

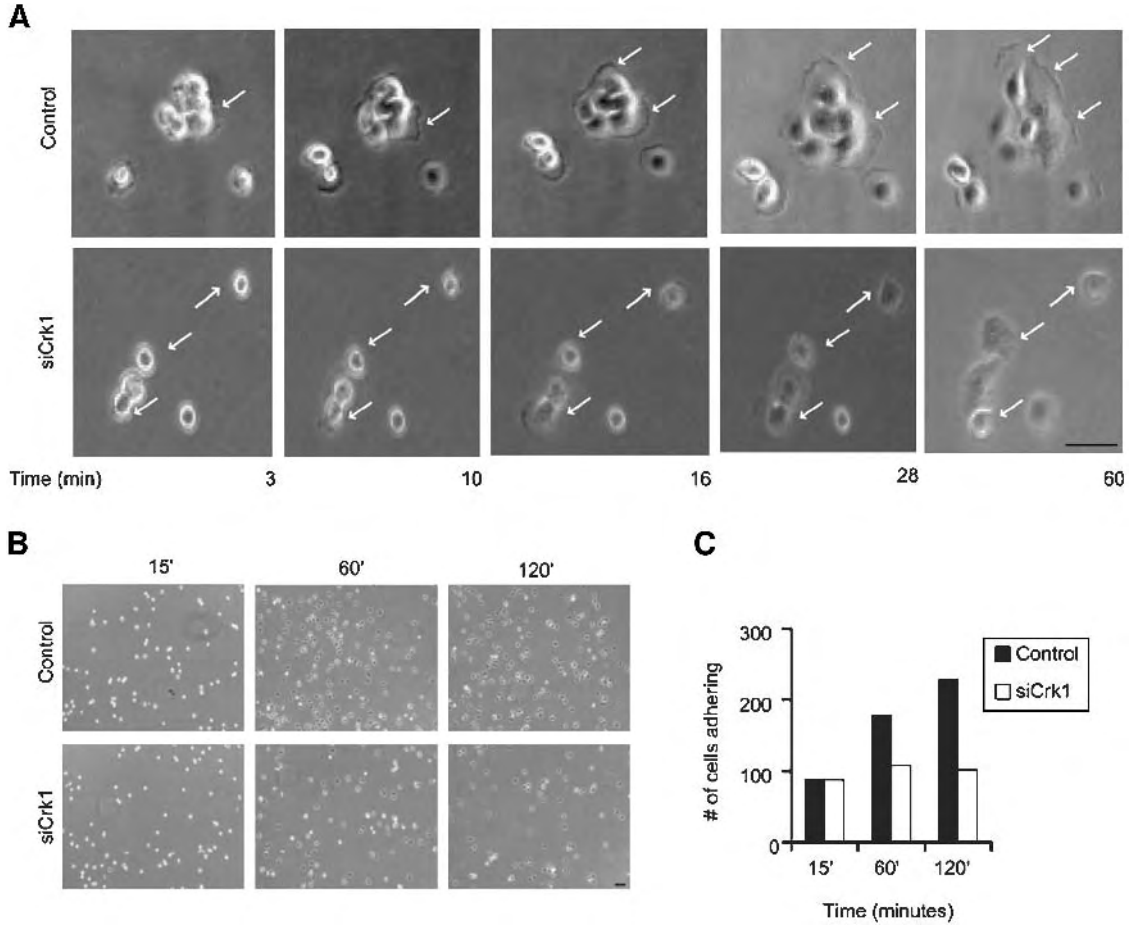

FIGURE 6. CrkI/II targeting siRNAs decrease cell adhesion and spreading. *White arrows,* lamellipodia and membrane protrusions in spreading cells. **A.** Time-lapse video-microscopy of MDA-231 cells transfected with CrkI/II siRNAs adhering to a fibronectin substrate. Forty hours posttransfection, cells were trypsinized and plated onto fibronectin-coated 35-mm plates and time-lapse video-microscopy was done using a Zeiss AxioVert 135 microscope and Northern Eclipse software. Frames were captured every 2 minutes. Transfection efficiency was analyzed by both immunofluorescence and Western blot analysis (data not shown). **B.** MDA-231 cells were transfected with siRNAs as previously described. Cells were trypsinized 48 hours posttransfection and replated on coverslips coated with 20 μg/mL fibronectin. Cells were washed and fixed in 4% paraformaldehyde at indicated time points. Images were captured using a Zeiss AxioVert 135 microscope and image analysis was carried out using Northern Eclipse software. **C.** Quantification of fixed time points in Fig. 6B). Cell numbers were acquired by manual counting using Northern Eclipse.

levels (siCrk2, Fig. 3A). Annexin V assays and proliferation counts confirmed that CrkI/II knockdown did not significantly enhance cell death or decrease cell proliferation throughout the duration of our assays (Fig. 3).

A major function assigned to CrkI and CrkII from overexpression studies is an enhanced signal for cell migration (6, 23, 25, 27). The effect of Crk knockdown on migration and invasion of single cells, as evaluated using Boyden chamber transwell assays, revealed a significant decrease in the migration and invasion capacity of various highly invasive human cancer cell lines as well as a decrease in HGF-enhanced migration (Figs. 4 and 5). These results are consistent with a role for Crk as a modulator of intracellular signals downstream from various growth factor receptors, such as the Met/HGF receptor (6, 37), vascular endothelial growth factor receptor (38), and ErbB2/Neu receptor (24), many of which are activated in human cancers (39). Although the signals that promote the invasive phenotype of these cell lines have not been characterized, these data show that Crk adaptor proteins play an important role in integrating signals for migration and invasion of highly migratory and invasive human cancer cell lines (Figs. 3-6).

Cell motility is a complex event that is dependent on the coordinated remodeling of the actin cytoskeleton and the regulated assembly and turnover of focal adhesions (for review see ref. 40). Crk knockdown resulted in decreased adhesion and spreading of cells as visualized by time-lapse microscopy (Fig. 6). Time-lapse microscopy of CrkI/II siRNA–transfected MDA-231 carcinoma cells further confirmed that a decrease in total levels of Crk proteins interferes with the ability of MDA-231 cells to form membrane protrusions, an event required during cell spreading, adhesion, and cell motility (Fig. 6). Such biological defects may result from the inability of these cells to sustain strong focal contacts with the extracellular matrix or to maintain actin polymerization at these sites.

Adhesion to the extracellular matrix is mediated at focal adhesion sites through clustering of integrins and cytoskeletal linking proteins, such as vinculin, filamin, α-actinin, actopaxin/parvin, and paxillin (reviewed in ref. 41). Paxillin localization at these sites is concomitant with the ability to form strong or stable focal adhesions required during cell migration and adhesion (4, 42-44). Crk adaptor proteins form a complex dependent on tyrosine phosphorylation with both paxillin and p130Cas, two proteins recruited to focal adhesions (4, 45, 46). Moreover, the overexpression of Crk enhances the formation of a complex of Crk with paxillin, Git2 and Pix, a Rac exchange factor, as well as elevated Rac activity in response to exogenous growth factor stimulation (4, 5). In support of reduced adhesion of Crk knockdown in MDA-231 cells, the analysis of endogenous paxillin and vinculin by confocal microscopy showed a decrease in the relocalization of paxillin and vinculin to focal adhesions, and a decrease in overall focal adhesions following the plating of cells on fibronectin (Fig. 7). Consistent with a reduced number of focal adhesions, we observed an overall decrease in bundled actin in cells transfected with siCrk (Fig. 7). Hence, knockdown of Crk proteins decreases the recruitment or maintenance of cytoskeletal proteins, such as paxillin, to focal adhesions, thereby reducing complexes required for the establishment of strong focal points and actin bundling. A similar phenotype was observed for paxillin- and p130Cas-null fibroblasts (42, 47, 48). In agreement with a role for Crk in the formation and/or maintenance of focal adhesions, epithelial Madin-Darby canine kidney cells that overexpress Crk contain enhanced numbers of focal adhesions (4).

The observation that cell migration and invasion of multiple human cancer cells is specifically decreased in the presence of Crk siRNA establishes Crk as a key integrator of signals involved in cell migration and invasion of highly invasive human cancer cells. This has important implications where elevated levels of Crk are observed in various human cancers, including glioma (26), lung cancer (28), as well as breast cancers (Fig. 1). In the breast cohort series, elevated Crk immunostaining was identified in breast adenocarcinoma and was evident in the cytoplasm and nucleus of the cancer cells (Fig. 1). Using this staining protocol, elevated Crk levels were detected in 60% of the tumors, which included stage I and stage III disease (Fig. 1). This supports the data of Miller et al. (28), who showed a similar relationship between Crk and lung cancer at the mRNA level. The relationship between elevated Crk and a worse overall survival in lung carcinoma patients could reflect a role for Crk in the development of a more clinically aggressive phenotype. Hence,

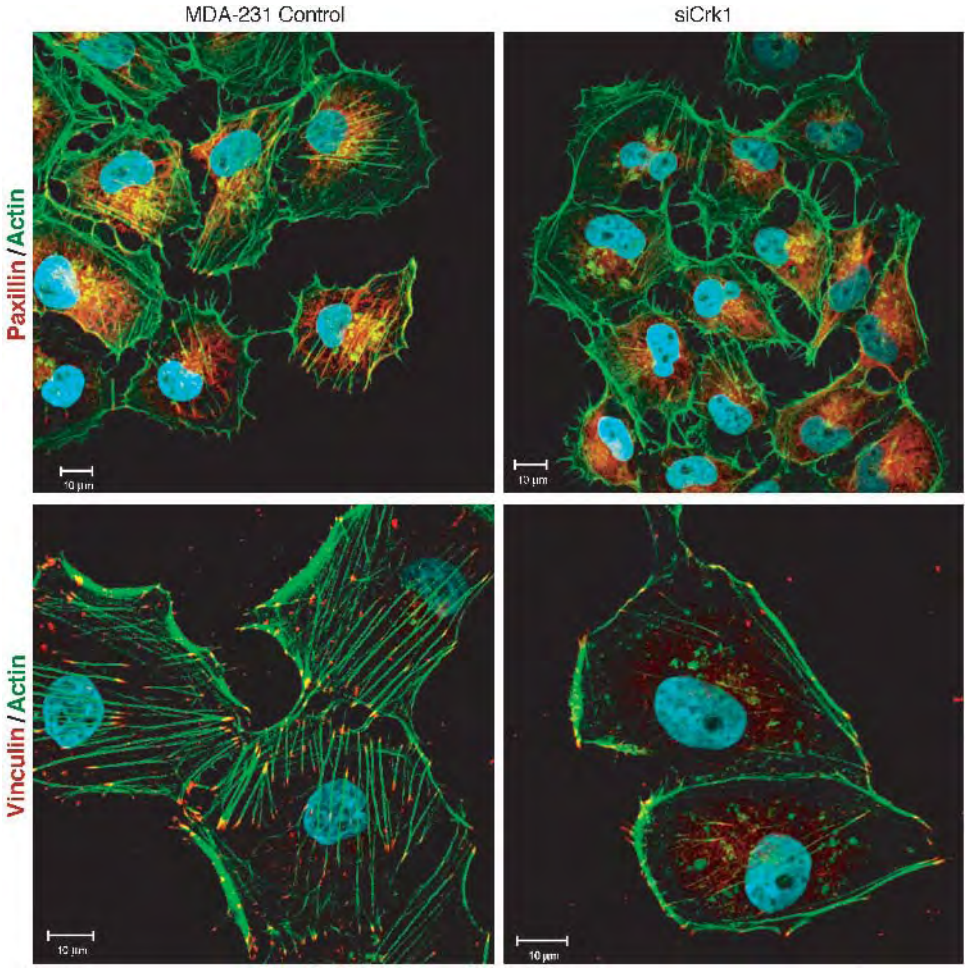

FIGURE 7. Knockdown of Crk proteins leads to decreases in focal adhesions and actin stress fibers. MDA-231 cells were transfected and replated on to fibronectin coated coverslips 48 hours posttransfection. Once fixed using 4% paraformaldehyde, cells were costained with α-paxillin/Alexa Fluor 555 goat anti-mouse and phalloidin-488 for the F-actin staining or α-vinculin/Alexa Fluor 555 goat anti-mouse and phalloidin-488. Images were acquired using a Zeiss confocal microscope. Actin and paxillin images were acquired using a 63× objective whereas the actin and vinculin images were obtained using a 100× objective. Bar, 10 μm.

our findings, demonstrating a role for Crk as a key integrator of signals for cell migration and invasion, identify a potential role for Crk in metastatic progression. Our results also highlight the need for further analysis of Crk status in human tumors.

Materials and Methods

Reagents and Antibodies

Monoclonal antibodies that recognize both CrkI and CrkII and monoclonal antibodies raised against paxillin were purchased from BD Transduction Laboratories (Lexington, KY). Antibodies against vinculin were purchased from Sigma-Aldrich (Oakville, Canada). Alexa Fluor 555-phalloidin as well as secondary goat antibodies against mouse conjugated Alexa Fluor 488 were purchased from Molecular Probes (Eugene, OR). Complete human cDNAs were purchased from Open-Biosystems (Huntsville, AL). The CrkII clone used, BC008506, was inserted in the mammalian expression vector pCMVSport6. The pOTB7-CrkI clone used was BC009837. Two siRNA duplex sequences were designed to target the *CRKI/II* gene. The siRNA sequences targeting the CrkI/II human mRNA corresponded to the coding region 264-284, 5′-AAUAGGA-GAUCAAGAGUUUGA-3′ and 5′-UCAAACUCUUGTU-CUCCUTUU-3′ [duplex previously published in Nagashima et al. (29)], or coding region 465-485, 5′-AGGAGACAUCUU-GAGAAUCdTdT-3′ and 5′-GAUUCUCAAGAUGUCUC-CUdTdT-3′ (designed using siRNA Design Center from Dharmacon Research, Inc., Lafayette, CO). The nontargeting RNA-induced silencing complex–free siRNA used as a control was designed by Dharmacon. This construct is chemically modified to impair processing by the RNA-induced silencing complex. All of the siRNA duplexes were synthesized, annealed, and purified by Dharmacon Research using 2′-ACE protection chemistry. Cy3 labeling of siRNAs was done using a Cy3-siRNA labeling kit (Ambion, Austin, TX).

Immunohistochemistry

Paraffin-embedded tissues were sectioned and pretreated in a microwave for 5 minutes for antigen retrieval. Immunostaining was done with a Ventana Immunostainer (Ventana Medical System, Inc., Tuscon AZ), using the monoclonal Crk antibody that recognizes both CrkI and CrkII isoforms, diluted 1:200 in EDTA (BD Transduction Laboratories) as the primary antibody. Peroxidase-labeled anti-rabbit immunoglobulin was used as secondary antibody and diaminobezidin as substrate. Each individual tumor was stained for Crk and scored for intensity (none, low, moderate, and high) separately by a pathologist and a surgeon. Controls of adjacent normal epithelium were used. A statistically significant difference was set at $P < 0.05$.

Western Blot

Cell lines were grown to 80% confluency and lysed in 1% Triton X-100 lysis buffer containing 50 mmol/L HEPES (pH 7.5), 150 mmol/L NaCl, 2 mmol/L EGTA, 1.5 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride, 1 mmol/L Na_3VO_4, 50 mmol/L NaF, 10 μg/mL aprotinin, and 10 μg/mL leupeptin. Whole cell lysates were resolved by SDS-PAGE in 12% gels. Western blot was done as previously described (49) by transferring the separated proteins onto a Hybond-enhanced chemiluminescence nitrocellulose membrane from Amersham Biosciences (Baie d'Urfe, Canada).

Cell Culture and Transfections

HeLa, H1299, KB, T47D, T47D-CrkII, and MDA-231 cells were maintained in culture in DMEM, whereas MDA-435s cells were maintained in Leibowitz media, all containing 10% fetal bovine serum and 50 μg/mL gentamicin (Invitrogen Canada, Inc., Burlington, Canada). Cells (3×10^4 cells/mL) were plated in 12-well plates 24 hours before transfection. A total of 100 pmol/L of siRNA was transfected per well using Lipofectamine and Plus reagents (Invitrogen) as per protocol of the company. Transfected cells were trypsinized and used in all assays 48 hours posttransfection. For immunofluorescence, 1.5×10^4 cells/mL were seeded on glass coverslips in 24-well plates and the amount of siRNA transfected per well was 60 pmol/L.

Migration and Invasion Assays

Cells (5×10^4) were counted and seeded directly onto 6.5-mm Corning Costar transwells for migration or transwells coated with 100 μg/cm^2 matrigel (BD Biosciences, San Jose, CA) for the invasion assays. Complete media was added to both the top and bottom wells and cells were incubated at 37°C overnight. For HGF stimulations, 34 ng/mL of HGF was added to the bottom wells. Following the overnight incubation, cells on both sides of the transwells were fixed using formalin phosphate for 20 minutes at room temperature. After washing with double-distilled water, cells were stained with 0.1% crystal violet in 20% methanol for 20 minutes at room temperature. Cells on the top layer were scraped and membranes were left to dry overnight. Images were captured using a Retiga 1300 digital camera (QIMAGING, Burnaby, Canada) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada Ltd., Toronto, Canada). Image analysis of these assays was carried out using Northern Eclipse version 6.0 (Empix Imaging, Mississauga, Canada) and quantification was done by using a constant threshold for all images and measuring pixels using Scion Image-NIH equivalent program for Microsoft Windows (Scion Company, Frederick, MD).

Adhesion and Spreading Assays

MDA-231 cells were plated on coverslips (for time point experiments) or 35-mm dishes (time lapse) previously coated with 20 μg/mL fibronectin (Ville Mont-Royal, Quebec, Canada) in PBS (for 30 minutes). Coverslips were washed with PBS at each time point and fixed. Images were acquired using a Retiga 1300 digital camera (QIMAGING) and a Zeiss AxioVert 135 microscope (Carl Zeiss Canada). Image analysis was carried out using Northern Eclipse version 6.0 (Empix Imaging). Time-lapse microscopy done during adhesion assays was captured using Northern Eclipse.

Indirect Immunofluorescence

Cells were fixed in 4% paraformaldehyde for 20 minutes and permeabilized using 0.2% Triton X-100 in PBS for 10 minutes. Coverslips were rinsed with 100 nmol/L glycine and blocked with 2% bovine serum albumin buffer (containing 0.2%

Triton X-100 and 0.05% Tween 20 in PBS) for 30 minutes. Primary antibodies diluted in blocking buffer were incubated for 1 hour. Paxillin antibody was diluted 1:200 and vinculin antibodies were diluted 1:400. All secondary antibodies were diluted 1:1,000 as well as Alexa 488-phalloidin. To counterstain nuclei, cells were incubated with 0.5 ng/mL 4′,6-diamidino-2-phenylindole for 5 minutes. Coverslips were mounted on glass slides using Immumount (Fisher Scientific, Nepean, Canada). All steps were carried out at room temperature. Images were acquired using a Retiga 1300 digital camera (QIMAGING) and a Zeiss confocal microscope.

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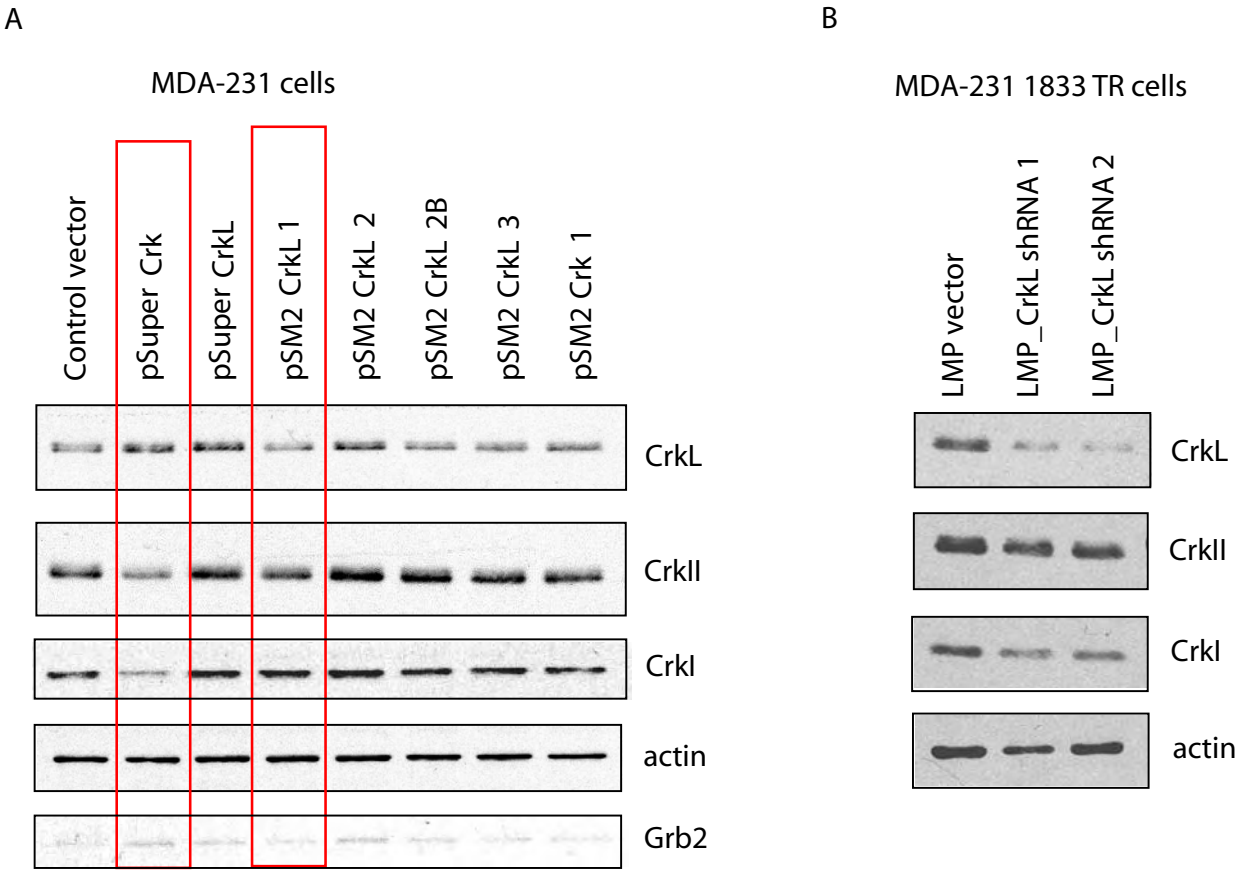
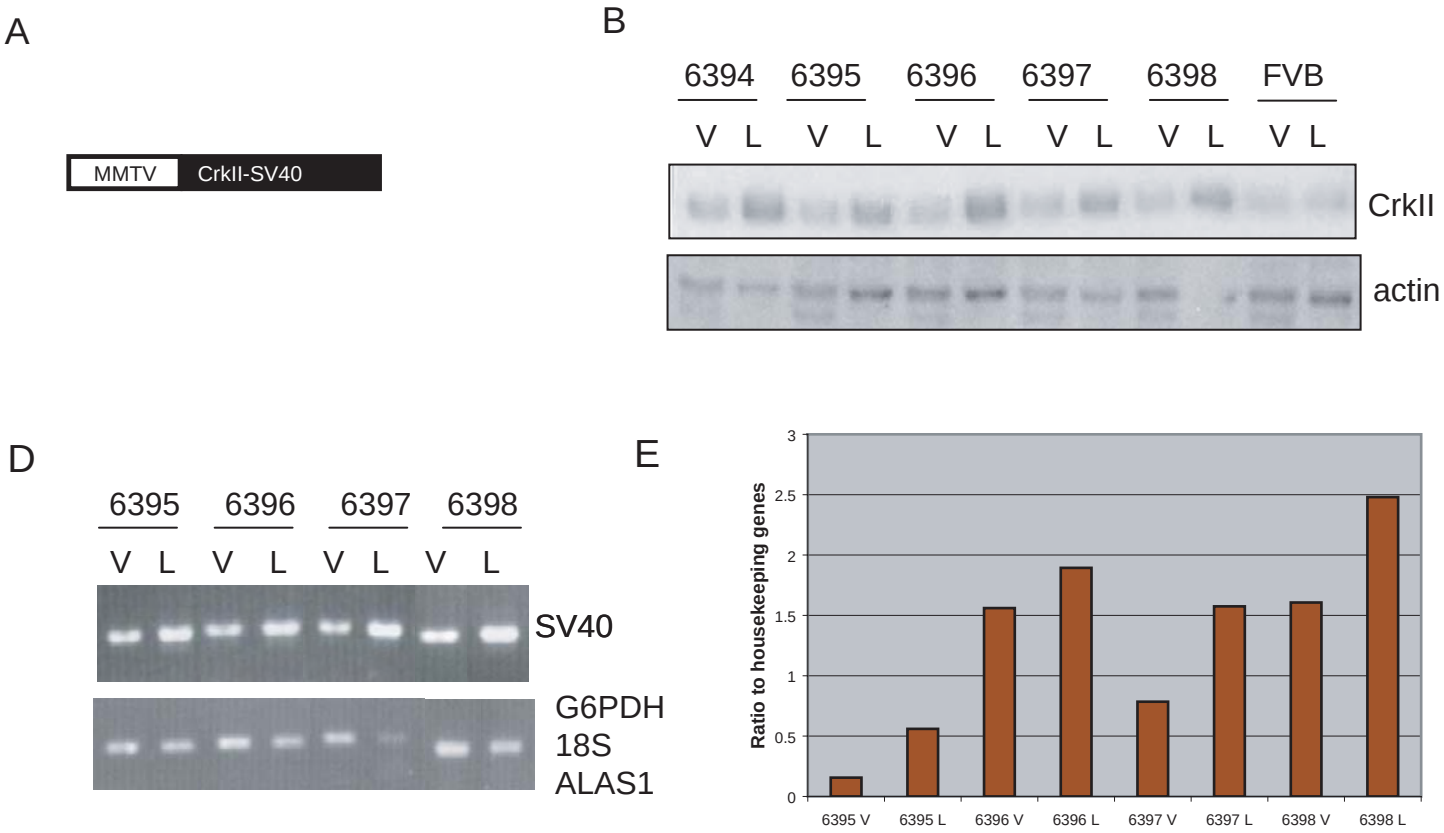
Cell Line	Migration	Invasion
MDA -231	80%	87%
MDA -435s	65%	70%
BT -549	12%	47%
BT -20	15%	31%

Appendix 2 - Knockdown of CrkI/II in "basal" human breast cancer cell lines. The percentage of inhibition in cellular migration and invasion relative to a mock transfected control for four different human breast cancer cell lines, described as being "basal" through gene expression profiling (9, 10).



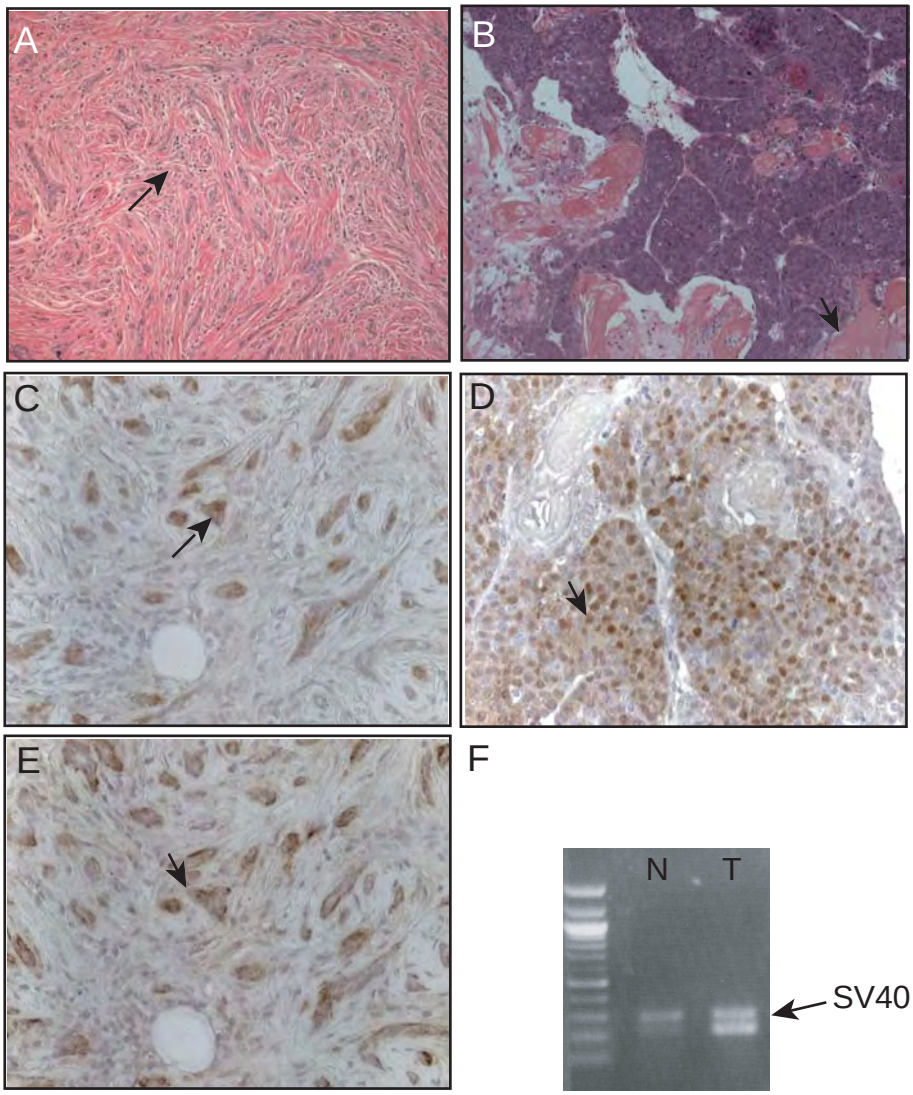
Appendix 3 - Stable knockdown of Crk adaptor proteins in the human breast cancer cell line MDA-231. (A) Compared to the pSuper empty vector, knockdown of CrkI/II is evident in the pSuper-CrkII stable cell line (derived from a whole cell population). Similar knockdown was not achieved for CrkL utilizing a pSuper-CrkL construct (also derived from a whole cell population). Individual clones from pSM2 constructs targeting CrkL (pSM2-CrkL 1, 2, 3) or CrkI/II (pSM2-Crk I) did not show significant decreases in the levels of their respective targets, except pSM2-CrkL1. Ten additional clones were tested in addition to the clones shown here. Knockdown of CrkII and CrkL are illustrated by the red boxes. No decrease in the level of Grb2 (another adaptor protein) was detected. (B) Whole cell populations of LMP, LMP_CrkL shRNA 1 and LMP_CrkL shRNA 2 show CrkL levels decreased in shRNA-containing MDA-231 1833TR cell lines.



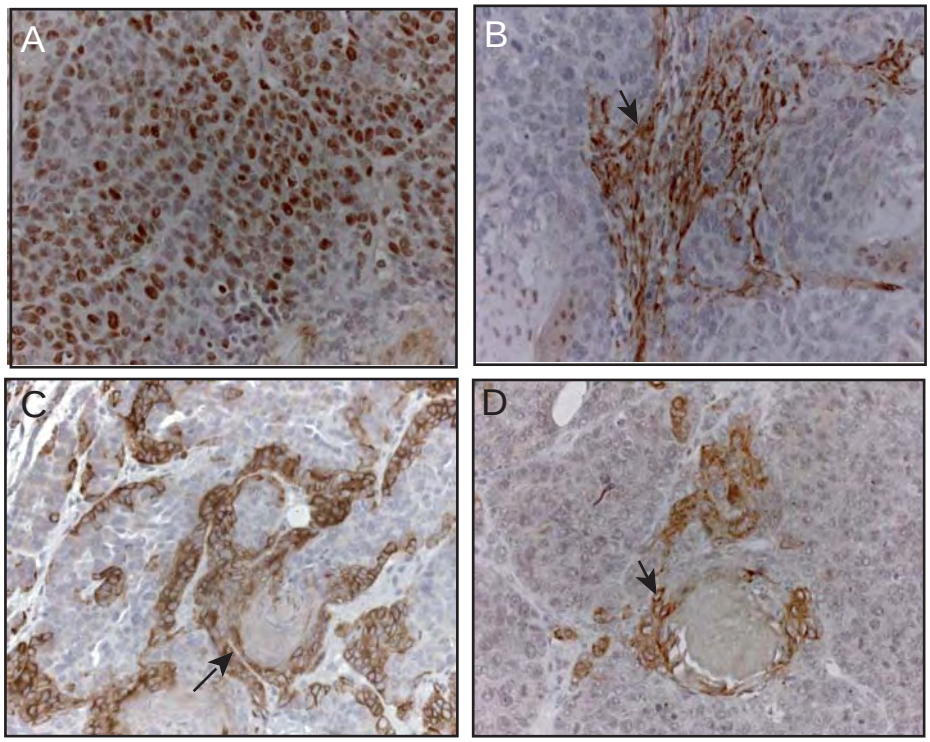
Appendix 4 - Expression of the CrkII transgene in the mammary epithelium. The design of the MMTV-CrkII transgene (A). Expression of CrkII protein in transgenic and FVB mice in both virgin (V) and lactating (L) animals (6394-6398 indicate the various transgenic mouse lines). Although the antibody recognizes endogenous protein, it is evident in lactating (and some virgin) animals that CrkII is higher in transgenic mice than wildtype FVB mice (C). Expression of transgene using primers specific for the SV40 region indicates the CrkII transgene is expressed well in each transgenic line. House keeping genes examined include G6PDH (shown here), 18S or ALAS1 (C). Semi-quantitative real-time PCR data illustrating the varying expression levels of the transgene in virgin and lactating female mice amongst the various transgenic lines (d). Immunohistochemistry of CrkII (ii, iii) in virgin transgenic mouse mammary epithelium compared to a normal FVB littermate (i) (F). Images i and iii taken at 40x, ii taken at 20x.

Line	# of glands analyzed		# of glands with tumors		Incidence per line	Combined incidence
	Virgin	Multiparous	Virgin	Multiparous		
6396	7	1	1	0	12.5	20.5
6398	5	2	1	1	28.6	
FVB	6	6	0	0	0	0

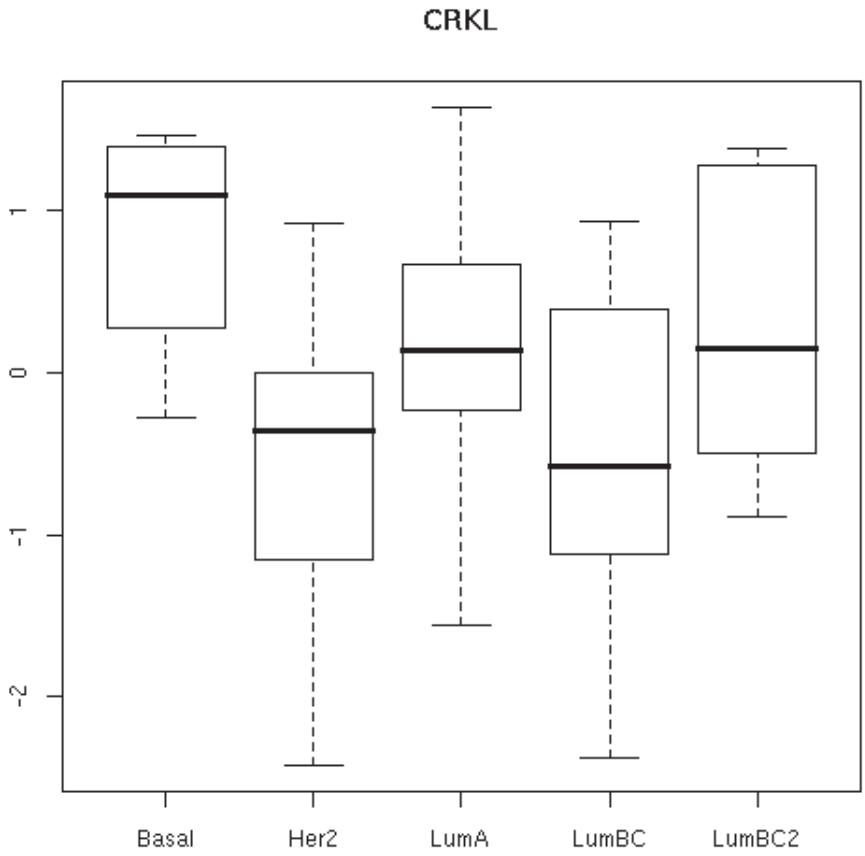
Appendix 5 - MMTV-CrkII tumor incidence. Mice from two MMTV-CrkII transgenic lines were set up into 2 groups - virgin or multiparous and monitored for tumor development (n=15 per virgin and multiparous). Preliminary results show that one multiparous female from line 6398 and two virgin mice (one from 6396, one from 6398) have each developed tumors. Each line (6396 and 6398) has a tumor incidence of 12.5% and 28.6% respectively, with a combined rate of 20.5%. No age-matched FVB have developed tumors thus far. The tumor latency was approximately 1 year of age.



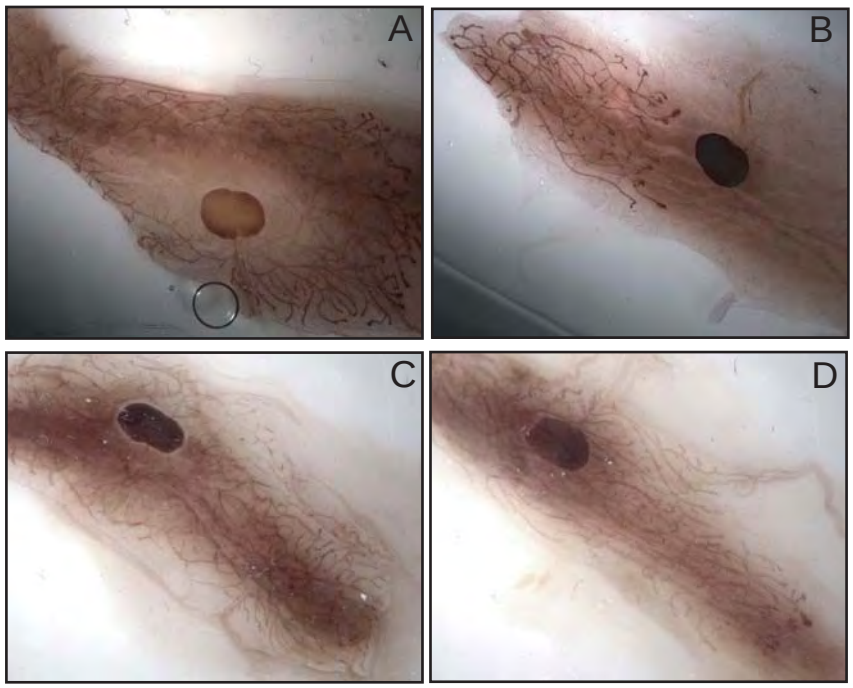
Appendix 6 - MMTV-CrkII over-expression leads to tumor development. A multi-focal spinde cell carcinoma in an aging virgin mouse at 446 days of age (A, 20x). A tumor found in mammary fat pad 2/3 in a multiparous female at 374 days classified as an adenosquamous carcinoma, characterized by the presence of cytokeratin pearls (B indicated by the arrow, 20x) and a high mitotic index. Both tumors over-expressed Crk protein (C, D, 40x) and staining for smooth muscle actin showed a similar staining pattern for the spindle cell carcinoma (E, 40x). Transgene expression for the adenosquamous carcinoma was confirmed by PCR analysis using primers specific for the transgene (N=normal mammary gland; T= tumor) (F).



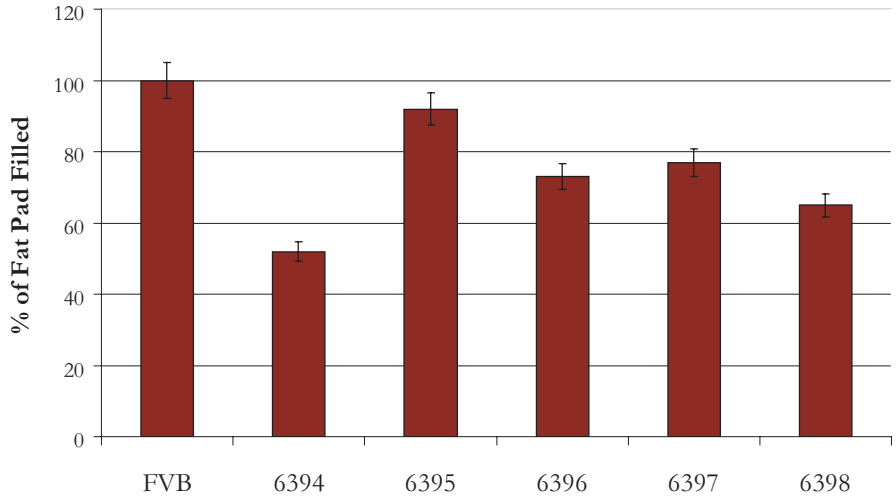
Appendix 7 - Characterization of the MMTV-CrkII squamous adenocarcinoma indicates it is highly proliferative and of mixed lineage. Staining for PCNA (A) indicates that the tumor is highly proliferative whereas staining for smooth muscle actin (B) indicates the potential presence of fibroblasts. CK14 (C) suggests that there is a high degree of myoepithelial cells present within the tumor. Staining for the putative progenitor marker CK6 (D) reveals that the MMTV-CrkII tumor may contain progenitor cells. All images taken at 40x.



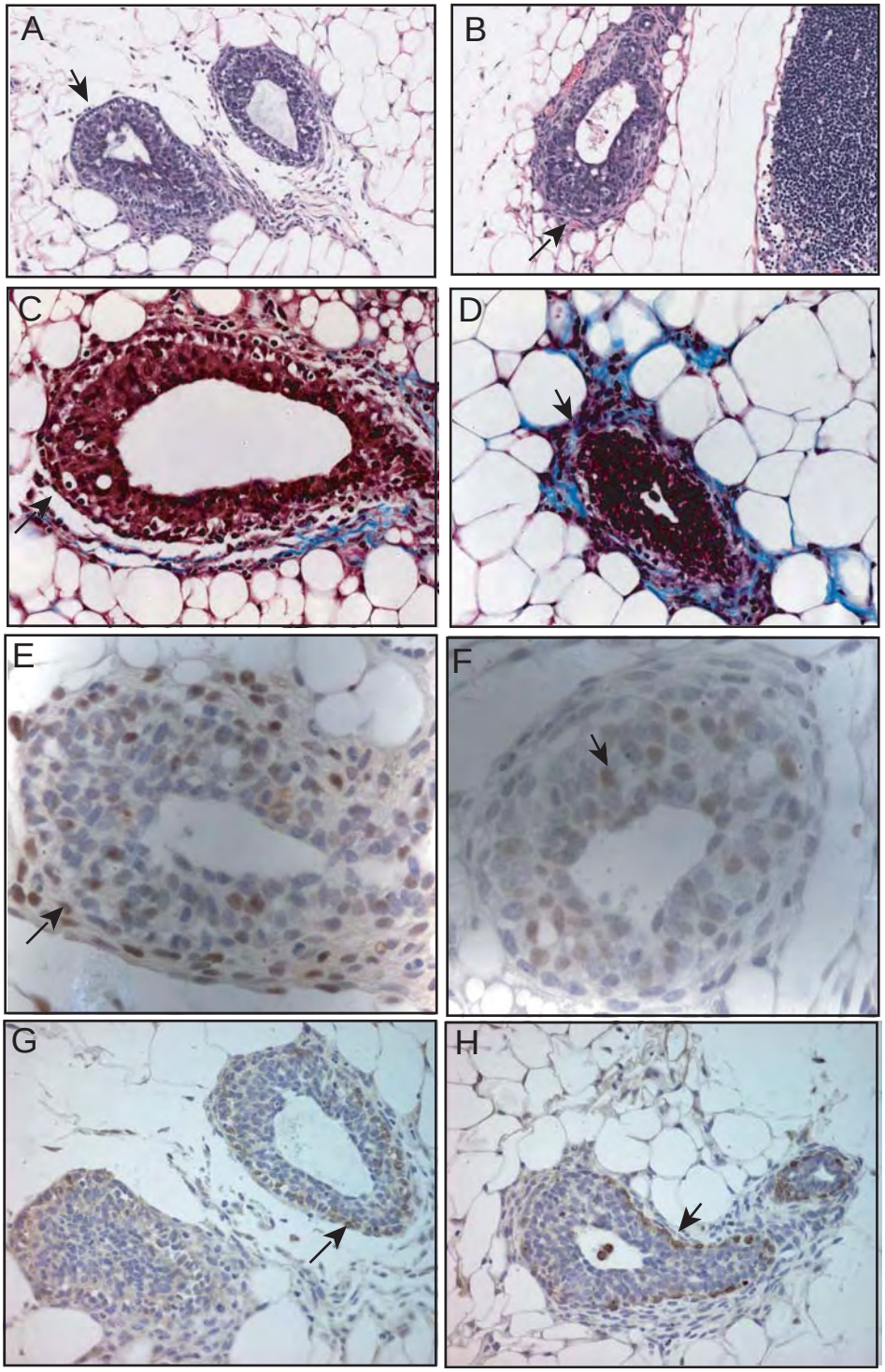
Appendix 8 - CrkL is at highest levels in basal human breast cancer. Gene expression profiling was carried out on breast cancer tissue derived from human patients. By examining probes specific for CrkL, it was found that CrkL is highly expressed in basal tumors (dark black bar represents the median) compared to other tumor types (Her2, Luminal subgroups A, BC, BC2) and normal epithelium.



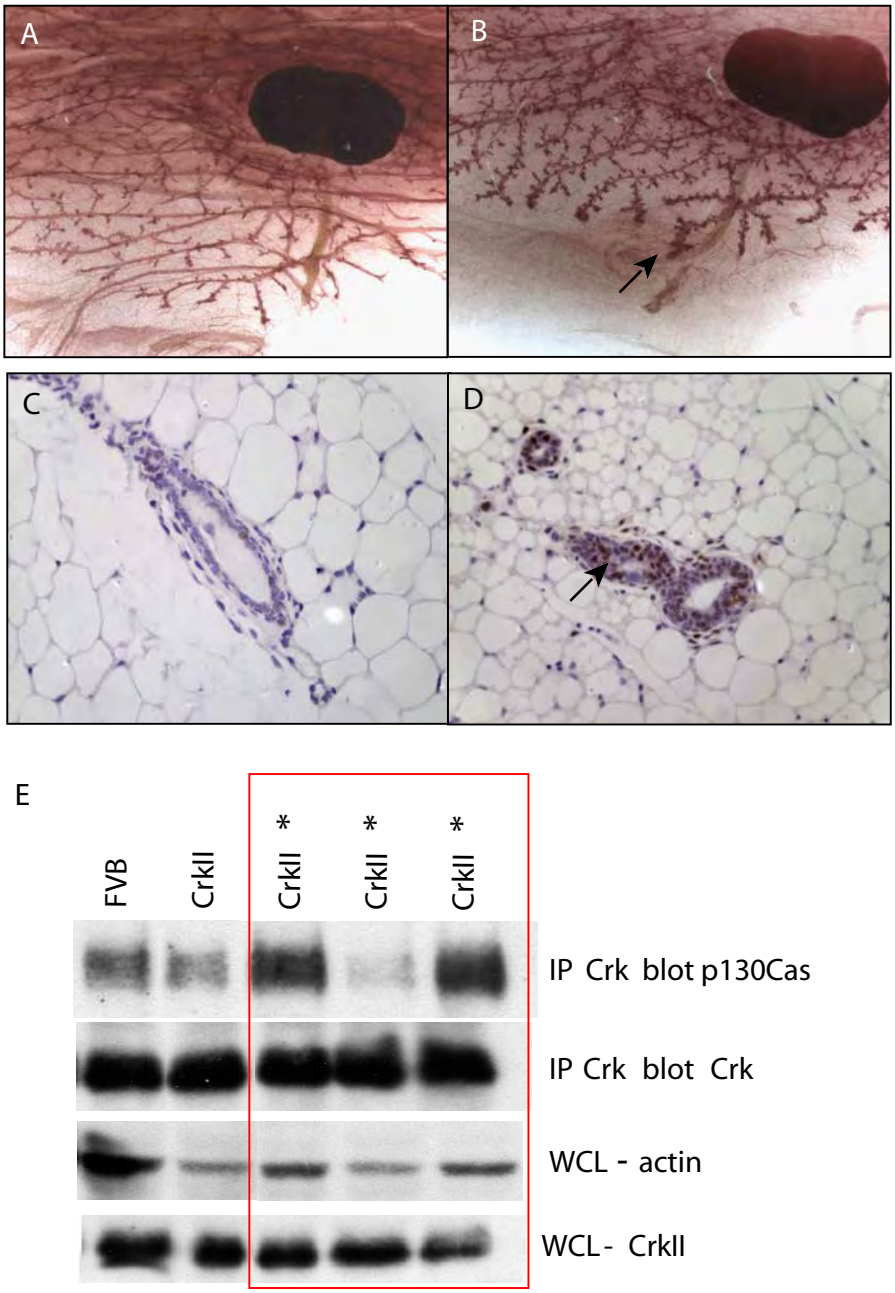
E



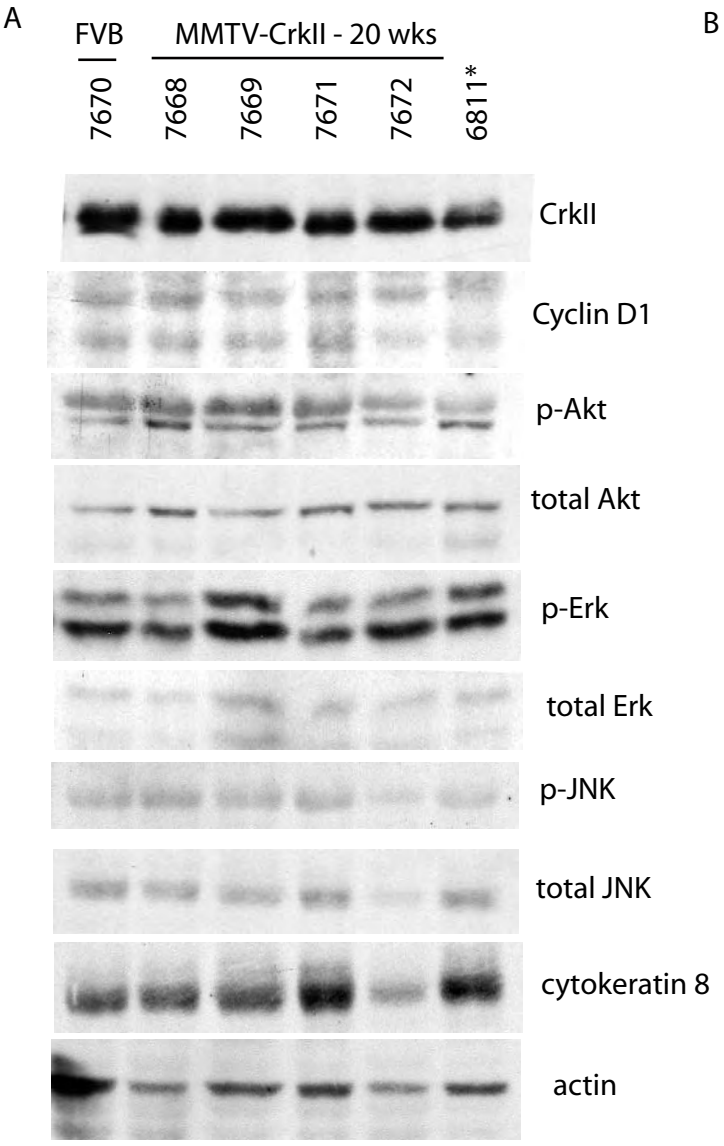
Appendix 9 - Delayed ductal outgrowth in MMTV-CrkII transgenic mice compared to normal FVB littermates. Delayed ductal outgrowth in transgenic mice was detected at 5 weeks (data not shown), 7 weeks (B) and 12 weeks (D) relative to FVB mice at the same age (A) and (C) respectively. Images A&B taken at 0.8x while C&D are taken at 0.6x. The percentage of fat pad filled by epithelial ducts was calculated using Scion Image for each mouse and the average percentage was determined for each transgenic line (6394-6398).

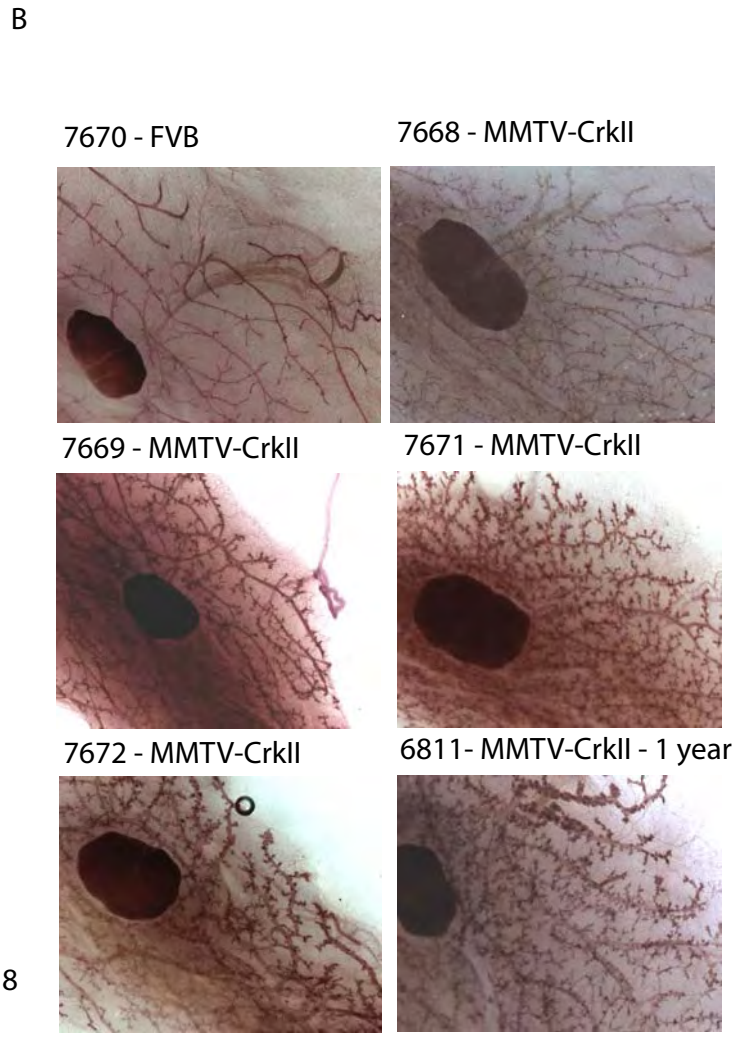


Appendix 10 - Comparison of FVB terminal end buds (A) to those from MMTV-CrkII transgenic mice (B) implies that CrkII terminal end buds have increased stroma and components of the basement membrane encompassing the end bud. Trichrome staining revealed that increased collagen surrounds the MMTV-CrkII transgenic end bud structures (D) but not wild-type terminal end buds (C). Staining for PCNA suggests that these structures are indeed proliferative (E). Preliminary staining indicates that CrkII is indeed expressed in the terminal end buds of transgenic mice (F) and that there is no difference in cytokeratin 14 expression between normal, FVB mice (G) and CrkII mice (H). All images taken at 40x, except A & B taken at 20x.

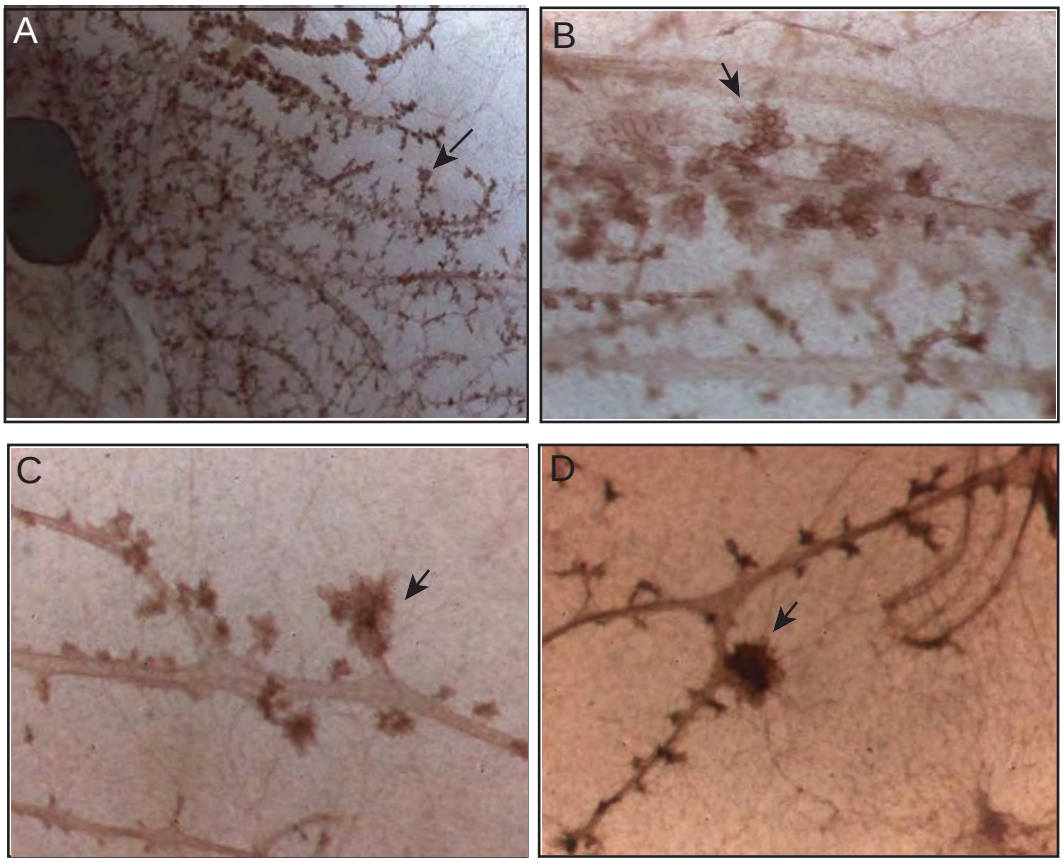


Appendix 11 - Enhanced branching of MMTV-CrkII mice at 20 weeks of age. In comparison to a wildtype littermate (A, 2x), MMTV-CrkII transgenic mice show enhanced branching (B, 2x). Staining for PCNA reveals active proliferation of the epithelial cells in MMTV-CrkII mammary ducts (D, 40x) whereas little to no proliferation is visualized in FVB mice (C, 40x). Co-immunoprecipitation of CrkII from 20 week old transgenic and FVB mice implies an increased p130Cas-CrkII association in CrkII mice with enhanced branching (red box) compared to normal FVB mice or MMTV-CrkII transgenic mice which do not show extensive side branching (E).

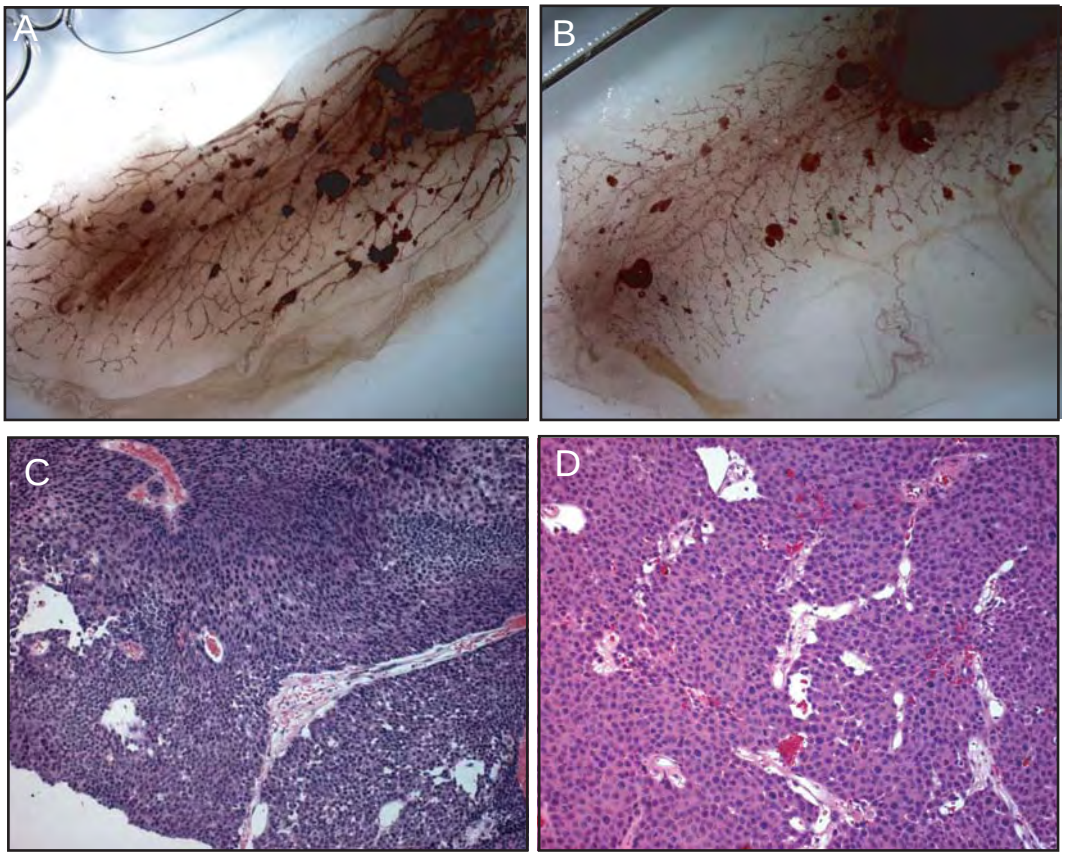


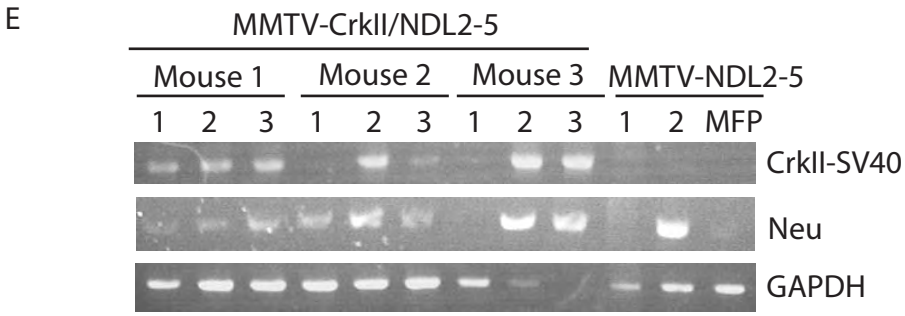


Appendix 12 - MMTV-CrkII enhanced branching does not significantly alter any major signaling pathways. Whole cell lysates from FVB or MMTV-CrkII mice at 20 weeks of age or 1 year of age were blotted for various signaling pathways to determine potential mechanisms for enhanced branching (A). No significant changes were visible in cyclin D1, Akt, Erk or JNK pathways. Cytokeratin 8 was used as an epithelial cell marker, whereas actin was used as a total cell lysate marker. The corresponding wholemount images show the variable branching of the MMTV-CrkII mice (B).

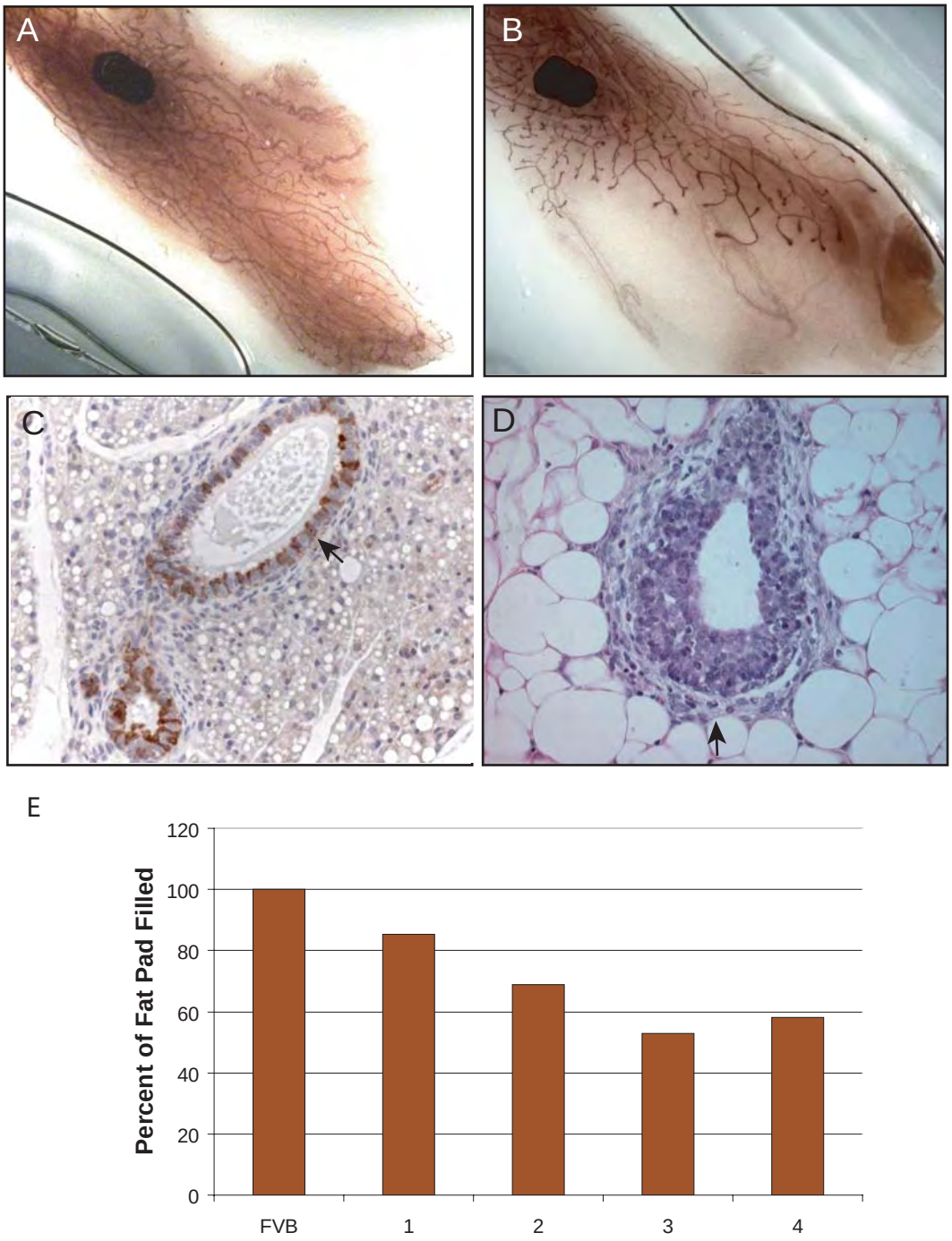


Appendix 13 - Aging virgin CrkII mice show a wide variety of phenotypes. Aging virgin mice sacrificed between 12-15 months display atypical epithelial architecture, such as enhanced branching (A, 2x), lobuloaveoli (B, 6.6x), spiculated ends (C, 6.6x) and potential hyperplasia (D, 6.6x).

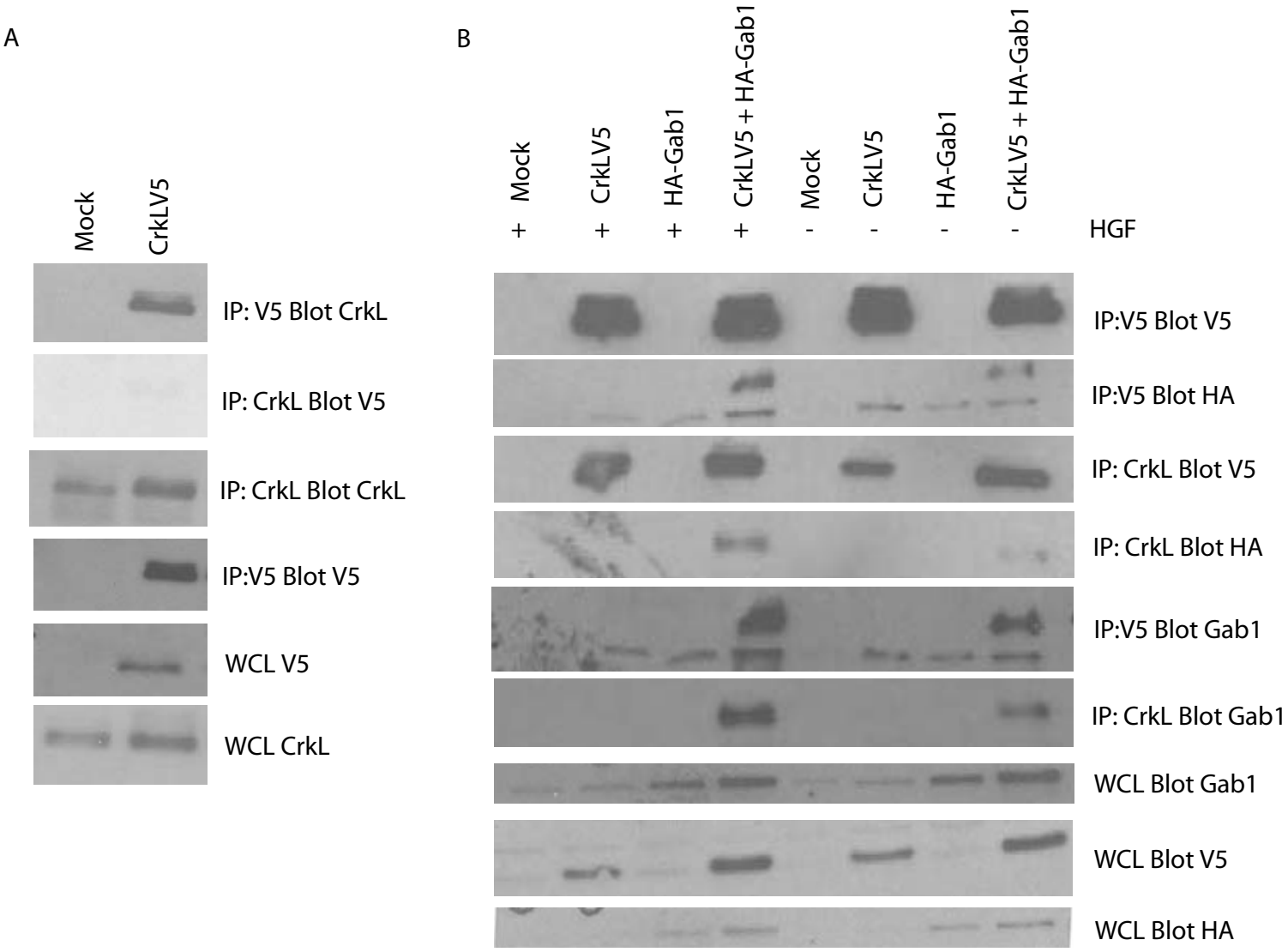




Appendix 14 - Over-expression of CrkII does not alter ErbB2 latency or tumor pathology. A comparison between MMTV-NDL2-5 (A, C) and MMTV-CrkII/NDL2-5 (B, D) shows no difference in tumor latency, degree of tumor development (as illustrated by wholemounts A and B, 0.8x) or tumor pathology (C and D, 40x). PCR on cDNA isolated from tumors indicates that CrkII-Neu tumours express both transgenes, whereas MMTV-NDL2-5 tumors only express Neu (E).



Appendix 15 - Delayed ductal outgrowth in MMTV-CrkI transgenic mice compared to normal FVB littermates. Delayed ductal outgrowth in transgenic mice was detected at 12 weeks (B) relative to FVB mice at the same age (A). Images A&B taken at 0.8x. CrkI expression is also evident in the mammary epithelium by immunostaining with an anti-mouse CrkI/II antibody and appears to be at cell-cell junctions (C, arrow 40x). Increased stroma appears to encompass the CrkI terminal end bud (D) at 40x. Four of 16 transgenic mice show delayed outgrowth at 12 weeks of age. The percentage of fat pad filled by epithelial ducts was calculated using Scion Image for each mouse and the average percentage was determined for each transgenic line which had delayed outgrowth (1-4) (E).



Appendix 16 - The CrkLV5 construct behaves similarly to endogenous CrkL and associates with Gab1 upon HGF stimulation. Co-immunopreciation of V5 and CrkL indicates that the V5 tag is specific to CrkL (A). CrkL-V5 can associate with its binding partner, HA-Gab1 and this association is enhanced upon HGF stimulation, similar to endogenous CrkL (15 minutes) (B).